Food Research

IN THIS ISSUE

SENSORY EVALUATION OF FOOD

Sequential Analysis in Organoleptic Research: Triangle, Paired, Duo-Trio Tests

MICROBIOLOGY OF FOOD

Some Fungi That Might Cause Softening of Storage Olives

NUTRITION

Nutritive Value Studies of a Wheat Flakes, Dried Whole Milk and Sugar Mixture

CHEMISTRY OF FOOD

Sampling Method for Analysis of Coffee Volatiles by Gas Chromatography

The Pigments of Off-Color Cooked Tuna Meat

Effect of Complexing and Chelating Agents on the After-Cooking Discoloration of Potatoes and upon the Iron and Phenolic Content of the Juice

Antioxidant and Copper Binding Properties of Onions

ENZYMOLOGY

Succinoxidase Activity of Some Animal Tissues

BACTERIOLOGY OF FOOD

Protective Role of Shell Membranes in Bacterial Spoilage of Eggs Microbial Flora of Packaged Frankfurters and Their Radiation Resistance

SANITATION ENGINEERING

Pretreatment of Brandy Stillage for Disposal in a Municipal Waste Treatment Plant

BIOCHEMISTRY OF FOOD

Aseptic Autolysis of Muscle: Biochemical and Microscopic Modifications Occurring in Rabbit and Lamb Muscle During Aseptic and Anaerobic Storage

NOTES AND LETTERS

Vitamin B_6 in Canned Pineapple Products

Volume 23, Number 3 *May-June, 1958*

AN OFFICIAL PUBLICATION OF

THE INSTITUTE OF FOOD TECHNOLOGISTS

Food Research

An Official Publication of

The Institute of Food Technologists

Volume 23 MAY-JUNE, 1958 Number 3

EDITORIAL BOARD

MARTIN S. PETERSON, *Editor-in-Chief*
(Editorial Office: 11606 South Bell Avenue,
Chicago 43, Illinois)

FRED E. DEATHERAGE
J. L. ETCHELLS
C. A. GREENLEAF
S. W. F. HANSON
MARGARET IVES
DANIEL MELNICK
R. T. MILNER
J. H. MITCHELL, JR.
HAROLD W. SCHULTZ
CHARLES R. STUMBO
REESE H. VAUGHN
R. O. WAGENAAR

Z. I. KERTESZ, *Consulting Editor*

CHARLES S. LAWRENCE, *Business Manager*
176 West Adams Street
Chicago 3, Illinois

Copyright, 1958
By the Institute of Food Technologists
Printed in U. S. A.

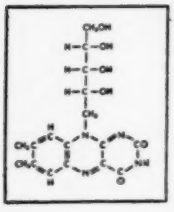
Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-522 N. Hickory Street, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

The Vital Story

A Quick History. Independent investigators, working separately to unlock several of nature's doors, sometimes open up unsuspected relationships. This happened with vitamin B_2.

Investigations. About 25 years ago, several groups, notably Warburg's, were investigating a "yellow enzyme" obtained from yeast. Almost simultaneously other investigators were studying a food factor that aided growth of laboratory animals.

What they found. Proceeding with chemical analysis of this growth factor, the team of Kuhn, György, and Wagner-Jauregg noted a relationship between the growth-producing agent and the "yellow enzyme." Their findings, and those of other researchers along similar lines, were published in 1933. Eventually, riboflavin and an essential part of the yellow enzyme were found to be identical and the unity of an essential nutrient and cellular metabolism was established.



Isolation of pure riboflavin was achieved by Kuhn and his co-workers, and by Ellinger and Koschara, in 1933.

Nomenclature. Known in the United States as riboflavin, this vitamin has also been called lactoflavin, ovoflavin, hepatoflavin, and vitamin G.

SYNTHESIS

By 1935, two eminent chemists, working separately, had synthesized riboflavin, practically in a dead heat. Prof. Paul Karrer of the University of Zurich, a collaborator of the Hoffmann-La Roche Laboratories, produced the first successful synthesis. Five weeks later Richard Kuhn of Germany announced his synthesis of the vitamin. Prof. Karrer subsequently shared the Nobel Prize in Chemistry for his work in vitamins and carotenoids.

The Karrer synthesis forms the basis for chemical processes in widespread use today by Hoffmann-La Roche and other leading manufacturers throughout the world. Riboflavin is also manufactured today by fermentation methods.

CHEMICAL AND PHYSICAL PROPERTIES

Riboflavin is yellow, slightly water-soluble with a greenish fluorescence and a bitter taste. Its empirical formula is $C_{17}H_{20}N_4O_6$. Vitamin B_2 produced by the Roche process is identical in every way with that occurring in nature.

How does vitamin B_2 work? Riboflavin is a vital part of nature's chain of reactions for utilization of carbohydrate energy. It has been found to be a constituent of many enzyme systems and is thus intimately connected with life processes. It is probably required by the metabolic processes of every animal and bird as well as by many fishes, insects and lower forms of life. (In certain animals, however, the requirement may be synthesized by bacteria within the intestine.)

In the cells riboflavin goes to work attached to a phosphate group. This substance, known as riboflavin-5′-phosphate or flavin mononucleotide, may in turn be attached to still another essential substance, adenylic acid, forming flavin adenine dinucleotide. Either nucleotide then is attached to protein, thereby forming an enzyme, and takes its part in oxidation-reduction reactions.

Requirements in Human Nutrition. As we have seen, vitamin B_2 is essential to life. We have no special storage organs in our bodies for this vitamin, although a certain level is maintained in various tissues, with relatively large amounts found in the liver and kidneys.

MEASURING METHODS

In the beginning, riboflavin activity was described in "Bourquin-Sherman units" and requirements were thought to be very small. Subsequent research showed otherwise. Milligrams of weight became the unit and the Food & Drug Administration of the U. S. Dept. of Health, Education & Welfare established (July 1, 1958) a minimum daily requirement of 1.2 mg. of riboflavin for all persons 12 or more years old. For infants it is 0.6 mg. These requirements are designed to prevent the occurrence of symptoms of riboflavin deficiency disease. The minimum daily requirement for this vitamin for children from 1 to 12 years is 0.9 milligram.

Recommended allowances. The Food & Nutrition Board of the National Research Council has recommended the following daily dietary allowances of riboflavin, expressed as milligrams. These are designed to maintain good nutrition of healthy persons in the U. S. A.

		Boys	Girls
Men		1.6	
Women		1.4	
"	(3rd trimester of pregnancy)	2.0	
"	(Lactating)	2.5	
Infants,	1-3 months	0.4	
"	4-9 "	0.7	
"	10-12 "	0.9	
Children,	1-3 years	1.0	
"	4-6 "	1.2	
"	7-9 "	1.5	
Adolescents,	10-12 years	1.8	1.8
"	13-15 "	2.1	2.0
"	16-20 "	2.5	1.9

# of VITAMIN B_2

(Riboflavin)

by Science Writer

Deficiencies of vitamin B_2 appear in several ways in human beings. The eyes, the skin, the nerves, and the blood show the effects of too little riboflavin. Laboratory animals have demonstrated that a riboflavin-deficient diet can cause death of adults and can slow or stop growth in the young. Female animals, deprived of riboflavin in the diet, may produce offspring with congenital malformations.

Medical uses. To overcome and control deficiencies in human beings, physicians have pure riboflavin available for administration by injection or orally, by itself or with other "B" vitamins or multi-vitamin-mineral combinations.

How do we get our daily riboflavin? Vitamin B_2 has wide distribution throughout the entire animal and vegetable kingdoms. Good sources are milk and its products, eggs, meats, legumes, green leaves and buds. Whole-grain cereals have significant but not large amounts of riboflavin.

ADDITION TO FOODS

Cereal foods play a large part in our diet. To produce the white flour almost all of us want, millers are obliged to remove parts of the wheat that contain much of the grain's riboflavin and other nutrients. In addition, cereal grains are not rich sources of riboflavin. Millers meet this problem by *enriching* the grain foods for which federal standards exist with vitamins B_1, B_2, niacin and the mineral iron. In the case of vitamin B_2, however, they do more than *restore* the processed food to its natural riboflavin level; they *fortify* the food with enough of this essential vitamin to make it nutritionally more valuable than it was in nature.

Acting to protect the good health of millions of Americans, bakers and millers adopted *enrichment* of white bread and white flour in 1941. Since that time, other foods, such as macaroni products, corn meal and grits, farina, pastina and breakfast cereals have had their food value increased by enrichment with pure riboflavin and other vitamins and minerals.

When enriching, fortifying or restoring, food manufacturers add the necessary quantity of riboflavin (and other vitamins and minerals) to the food during processing, so that the finished product meets federal, state, and territorial requirements or contributes to the consumer an amount of the vitamin that dietary experts believe significantly useful.

PRODUCTION

Prof. Karrer's synthesis of riboflavin was a laboratory success. Adapting the process to commercial production, however, demanded original thinking by chemists at Hoffmann-La Roche. The production of riboflavin by chemical synthesis requires the production of ribose, a rare sugar, at an early stage in the process. This special sugar must be made inexpensively if the synthesis is to be practical. Sugar chemistry is a difficult matter. In a brilliant piece of work, the Roche chemical experts developed a method to produce ribose on a commercial scale by an electrolytic process, thus overcoming a most troublesome problem. Subsequently, Roche chemists developed the first practical synthesis for riboflavin-5′-phosphate, identical with natural flavin mononucleotide.

Picture three streams joining to form a river and you have a simplified idea of the Roche process for synthesizing vitamin B_2. O-xylene and glucose are processed separately to form xylidine and ribose respectively. These are joined to form ribitylxylidine, which is then converted to ribitylaminoxylidine. Starting separately with malonic ester, which is processed through intermediate stages to alloxan, the third "stream" is then joined with ribitylaminoxylidine to form riboflavin. Purification occurs at each step of the synthesis. Riboflavin 'Roche' equals or exceeds U. S. P. standards.

By the tons. So efficient is the Roche process that pure riboflavin is produced *by the tons* for use in pharmaceutical products and processed foods. An interesting development by Roche is the production of riboflavin in different forms related to the method of end use. 'Roche' Regular riboflavin U. S. P. is especially useful in dry enrichment premixes, powdered dietary supplements, pharmaceutical tablets and soft gelatin capsules. 'Roche' Solutions type is preferred for the manufacture of solutions having low concentration. 'Roche' Riboflavin-5′-Phosphate Sodium is a highly and rapidly soluble riboflavin compound favored for all pharmaceutical liquid products and some tablets, lozenges, and capsules. It has a more pleasant taste than the bitter U. S. P. riboflavin.

This article is published in the interests of pharmaceutical manufacturers, and of food processors who make their good foods better using pure riboflavin 'Roche.' Reprints of this and others in the series will be supplied on request without charge. Also available without cost is a brochure describing the enrichment or fortification of cereal grain products with essential vitamins and minerals. These articles and the brochure have been found most helpful as sources of accurate information in brief form. Teachers especially find them useful in education. Regardless of your occupation, feel free to write for them. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon St., St. Laurent, P. Q.

Volume 23 MAY-JUNE, 1958 Number 3

CONTENTS

SENSORY EVALUATION OF FOOD

SEQUENTIAL ANALYSIS IN ORGANOLEPTIC RESEARCH: TRIANGLE, PAIRED, DUO-TRIO TESTS. Andrew P. Radkins 225

MICROBIOLOGY OF FOOD

SOME FUNGI THAT MIGHT CAUSE SOFTENING OF STORAGE OLIVES. George D. Balatsouras and Reese H. Vaughn 235

NUTRITION

NUTRITIVE VALUE STUDIES OF A WHEAT FLAKES, DRIED WHOLE MILK AND SUGAR MIXTURE. G. H. Reussner, Jr. and R. Thiessen, Jr. 244

CHEMISTRY OF FOOD

SAMPLING METHOD FOR ANALYSIS OF COFFEE VOLATILES BY GAS CHROMATOGRAPHY. John W. Rhoades 254

THE PIGMENTS OF OFF-COLOR COOKED TUNA MEAT. W. Duane Brown, A. L. Tappel, and H. S. Olcott 262

EFFECT OF COMPLEXING AND CHELATING AGENTS ON THE AFTER-COOKING DISCOLORATION OF POTATOES AND UPON THE IRON AND PHENOLIC CONTENT OF THE JUICE. Mercedes L. Hunsader and Flora Hanning 269

ANTIOXIDANT AND COPPER BINDING PROPERTIES OF ONIONS. Emma Jo Lewis and Betty M. Watts 274

ENZYMOLOGY

SUCCINOXIDASE ACTIVITY OF SOME ANIMAL TISSUES A. L. Tappel and Ruth Martin 280

BACTERIOLOGY OF FOOD

PROTECTIVE ROLE OF SHELL MEMBRANES IN BACTERIAL SPOILAGE OF EGGS. J. A. Garibaldi and J. L. Stokes 283

MICROBIAL FLORA OF PACKAGED FRANKFURTERS AND THEIR RADIATION RESISTANCE. Suzanne Dewees Drake, James B. Evans, and C. F. Niven, Jr. . 291

SANITATION ENGINEERING

PRETREATMENT OF BRANDY STILLAGE FOR DISPOSAL IN A MUNICIPAL WASTE TREATMENT PLANT. Reese H. Vaughn, George K. York II, Melvin F. Klemmick, and L. Douglas Davis 297

BIOCHEMISTRY OF FOOD

ASEPTIC AUTOLYSIS OF MUSCLE: BIOCHEMICAL AND MICROSCOPIC MODIFICATIONS OCCURRING IN RABBIT AND LAMB MUSCLE DURING ASEPTIC AND ANAEROBIC STORAGE. R. Zender, C.-Lataste-Dorolle, R. A. Collet, P. Rowinski, and R. F. Mouton 305

NOTES AND LETTERS

VITAMIN B_6 IN CANNED PINEAPPLE PRODUCTS. L. J. Teply 327

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $7.50 per volume.

Non-members: Domestic, $8.50 per volume; foreign, $10.00 per volume.

Subscriptions and renewals are entered to begin with the first issue of the current volume.

Single copies of current volume: Domestic, $1.50; foreign, $1.75.

CORRESPONDENCE

Editorial: MARTIN S. PETERSON, Editor-in-Chief, 11606 South Bell Ave., Chicago 43, Ill.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: FOOD RESEARCH, 510-522 N. Hickory Street, Champaign, Ill.

Member Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.

Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in FOOD RESEARCH. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for FOOD RESEARCH should conform to the style used in the journal and be submitted in duplicate to the Editor-in-Chief. Authors of papers intended for FOOD RESEARCH should request from the Editorial office a copy of the booklet "Information and suggestions for authors." FOOD RESEARCH reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

SEQUENTIAL ANALYSIS IN ORGANOLEPTIC RESEARCH: TRIANGLE, PAIRED, DUO-TRIO TESTS

ANDREW P. RADKINS
Purdue University, Lafayette, Indiana

(Manuscript received September 20, 1957)

It is the purpose of this paper to explain and demonstrate a different approach to testing the statistical hypotheses associated with triangle, paired, and duo-trio tests. This suggested approach makes use of sequential analysis.

The literature of the last 9 years indicates a steady improvement in hypothesis testing methods associated with organoleptic research. For example, in 1948, Roessler, Warren, and Guymon *(7)* published an hypothesis testing method applicable to the triangle test; however, this method did not include consideration of one versus two-tailed hypothesis tests, nor did it include consideration of the type II error. Since then, both of these omitted points have been covered. Roessler, Baker, and Amerine *(6)* give clear examples demonstrating the type of experimental interest dictating a one or a two-tailed hypothesis test; this article also includes tables for testing triangle ranking and paired test hypotheses. The meaning and importance of the type II error in organoleptic research is discussed by Baker, Amerine, and Roessler *(1)*; they do not present a method for testing hypotheses incorporating the type II error. However, a method has been suggested by Radkins *(5)* which allows the experimenter to test various organoleptic hypotheses considering both the type I and type II errors and the use of either a one or a two-tailed critical region.

All of these hypothesis testing methods, ignoring their relative merits, have one thing in common. They are fixed sample size tests. In using the hypothesis testing methods not including consideration of the type II error *(6, 7)*, the experimenter arbitrarily decides on a suitable number of observations to take and determines, by solving a formula or entering a table, the critical region based on α, his choice of the probability of committing the type I error. The experiment is performed by gathering the number of observations decided upon; the hypothesis is accepted or rejected by comparing the observed results to the critical region. In the procedure mentioned previously as incorporating the type II error *(5)*, a calculated but fixed number of observations are taken, and the hypothesis is accepted or rejected by comparing the observed results to the calculated critical region. Thus the procedures reviewed here for testing organoleptic hypotheses commit the experimenter to a fixed sample size.

The essential difference between the hypothesis testing methods reviewed so far and the sequential analysis approach is that sequential analysis allows accepting or rejecting the hypothesis with a variable sample size.

PLANNING EXPERIMENTS USING SEQUENTIAL ANALYSIS

Consider a triangle test example; perhaps the experimenter is interested in determining whether a substitute raw material produces a detectable difference in the finished product. The null hypothesis would appropriately state there is no difference between finished products made with regular and

substitute raw materials. This hypothesis can be stated statistically as

$$p'_1 = 0.33.$$

The symbol p'_1 is the expected proportion of trials or triangle presentations in which the odd sample is correctly identified. The alternate hypothesis would appropriately state there is a difference in finished products; statistically stated the alternate would be

$$p'_2 > 0.33.$$

In this case p'_2 is the expected proportion of trials or triangle presentations in which the odd sample is correctly identified. The definitions for p'_1 and p'_2 are identical; the subscripts 1 and 2 on p' differentiate between expected proportions under the hypothesis and the alternate hypothesis, respectively. Note that this alternate hypothesis has many values; the choice of a specific value for p'_2 is up to the experimenter. A value of 0.34 or 0.35 for p'_2 would rarely be different enough from the p'_1 of 0.33 to be of practical interest. In this example, the specific alternate hypothesis used will be

$$p'_2 = 0.43.$$

The difference between the proportions from the hypothesis and the alternate hypothesis large enough to be considered a practical or important difference has been labelled δ *(3)*. In other words, in this example δ is equal to 0.10.

Next, the experimenter arbitrarily chooses α and β risks. The α risk is the probability of committing a type I error; that is, the probability of rejecting the hypothesis when in fact the hypothesis is true. The β risk is is the probability of committing a type II error; that is, the probability of accepting the hypothesis when in fact the alternate hypothesis is true. Here $\alpha = 0.05$ and $\beta = 0.10$ will be used.

Summarizing up to this point, the hypothesis has been stated, a value for δ has been chosen allowing a specific alternate hypothesis to be stated, and the α and β risks have been specified. These steps are exactly the same steps an experimenter would go through using a comparable fixed sample size approach.

From here on, the sequential analysis approach is different. The sequential analysis approach used here is from Burr *(2)*. There are two formulas which make up the hypothesis testing decision rule. The first formula

$$D_o \leqslant -h_1 + SN \tag{1}$$

defines the region in which the hypothesis is accepted. In this formula in the current context, D_o is the observed number of triangle presentations in which the odd sample was correctly identified, and N is the sample size or the number of triangles presented. Formulas are available for h_1 and S in terms of the hypothesized proportions, p'_1 and p'_2, and the α and β values:

$$h_1 = \left[\log \frac{1-\alpha}{\beta}\right] \Big/ \left[\log \frac{p'_2\,(1-p'_1)}{p'_1\,(1-p'_2)}\right] \tag{2}$$

$$S = \left[\log \frac{1-p'_1}{1-p'_2}\right] \Big/ \left[\log \frac{p'_2\,(1-p'_1)}{p'_1\,(1-p'_2)}\right]$$

These formulas look far more difficult to work with than they actually are. For example, equation 2 is solved by substituting the appropriate values as follows.

$$\begin{aligned} h_1 &= \left[\log \frac{1-\alpha}{\beta}\right] \Big/ \left[\log \frac{p'_2\,(1-p'_1)}{p'_1\,(1-p'_2)}\right] \\ &= \left[\log \frac{1-0.05}{0.10}\right] \Big/ \left[\log \frac{0.43\,(1-0.33)}{0.33\,(1-0.43)}\right] \\ &= \log 9.500 \;/\; \log 1.532 \\ &= 0.97772 \;/\; 0.18526 \\ &= 5.28 \end{aligned} \tag{2}$$

Similarly equation 3 is solved for S by substitution. The denominator in equation 3 for S is identical to the denominator in equation 2 for h_1; this is a point which saves considerable computational effort.

$$\begin{aligned} S &= \left[\log \frac{1-p'_1}{1-p'_2}\right] \Big/ \left[\log \frac{p'_2\,(1-p'_1)}{p'_1\,(1-p'_2)}\right] \\ &= \left[\log \frac{1-0.33}{1-0.43}\right] \Big/ \left[\log \frac{0.43\,(1-0.33)}{0.33\,(1-0.43)}\right] \\ &= \log 1.175 \;/\; \log 1.532 \\ &= 0.07004 \;/\; 0.18526 \\ &= 0.38 \end{aligned} \tag{3}$$

The values so obtained for h_1 and S are put into inequation or formula 1 giving

$$D_o \leqslant -5.28 + 0.38N. \tag{4}$$

The term inequation is appropriate here because the *less than* sign and the *equal to* sign are both required.

Thus after N triangle trials have been conducted, if the odd sample was correctly identified on $-5.28 + 0.38$ N or fewer trials, accept the hypothesis $p'_1 = 0.33$. It is perhaps disconcerting that a decision concerning a proportion hypothesis, $p'_1 = 0.33$, is being made in terms of numbers of trials; inequations 1 and 4 could be transformed into terms of proportions by dividing both sides by N. If this were done, the inequations would no longer be linear; this is a point of practical importance in graphing the sequential analysis rule as shall be demonstrated later.

The second formula making up the hypothesis testing decision rule is

$$D_o \geqslant h_2 + SN. \tag{5}$$

This formula defines the region in which the hypothesis is rejected. As in inequation 1, D_o is the observed number of triangle presentations in which the odd sample was correctly identified, and N is the sample size or the number of triangles presented. Here again formulas are available for h_2 and S in terms of the hypothesized proportions, p'_1 and p'_2, and the α and β values:

$$h_2 = \left[\log \frac{1-\beta}{\alpha}\right] \Big/ \left[\log \frac{p'_2\,(1-p'_1)}{p'_1\,(1-p'_2)}\right] \tag{6}$$

$$S = \left[\log \frac{1 - p'_1}{1 - p'_2}\right] \Big/ \left[\log \frac{p'_2\,(1 - p'_1)}{p'_1\,(1 - p'_2)}\right] \tag{3}$$

Here the formula for S is exactly the same as the formula for the S used in inequation 1 so solving for S is unnecessary here. Equation 6, the denominator of which has already been evaluated, is solved for h_2 by making the appropriate substitutions as follows:

$$\begin{aligned} h_2 &= \left[\log \frac{1 - \beta}{\alpha}\right] \Big/ \left[\log \frac{p'_2\,(1 - p'_1)}{p'_1\,(1 - p'_2)}\right] \\ &= \left[\log \frac{1 - 0.10}{0.05}\right] \Big/ \left[\log \frac{0.43\,(1 - 0.33)}{0.33\,(1 - 0.43)}\right] \\ &= \log 18.00 \;/\; \log 1.532 \\ &= 1.25527 \;/\; 0.18526 \\ &= 6.78 \end{aligned} \tag{6}$$

Using the computed values for h_2 and S, inequation 5 becomes

$$D_o \geqslant 6.78 + 0.38\,N \tag{7}$$

Thus after N triangle trials have been conducted, if the odd sample was correctly identified on $6.78 + 0.38\,N$ or more trials, reject the hypothesis $p'_1 = 0.33$ and assume the alternate hypothesis $p'_2 = 0.43$ is true.

Figure 1 graphically describes the hypothesis testing decision rule that has been developed for a triangle test with the hypothesis $p'_1 = 0.33$, the alternate hypothesis $p'_2 = 0.43$, $\alpha = 0.05$, and $\beta = 0.10$. The two lines on Figure 1 are formed by considering inequations 4 and 7 as exact equalities. Inequation 4 reads, if D_o, the observed number of triangles for which the odd sample is correctly identified, is less than or equal to $-5.28 + 0.38\,N$, accept the hypothesis. Thus because the inequation reads less than, the area below $D_o = -5.28 + 0.38\,N$ is labelled the accept region. Recognizing that inequation 7 reads greater than or equal to, the area above $D_o = 6.78 + 0.38\,N$ is

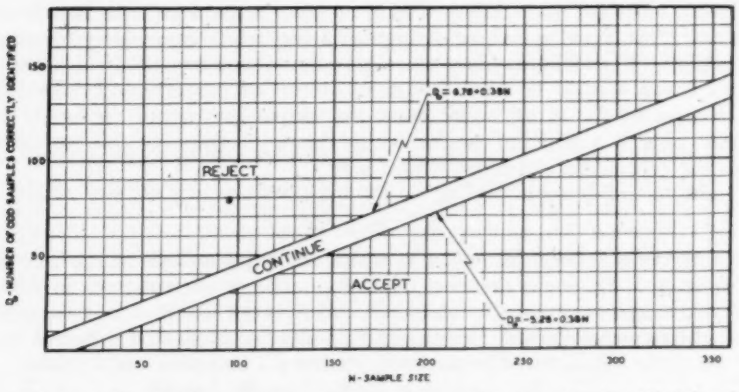


Figure 1. Sample size versus number of odd samples correctly identified for the hypothesis $p'_1 = 0.33$, alternate $p'_2 = 0.43$, $\alpha = 0.05$, $\beta = 0.10$.

appropriately labelled the reject region. The area between the accept and reject lines calls for continuing the experiment; the fact that the continue region is formed by two parallel lines extending indefinitely probably concerns the reader. That this concern is unwarranted will be treated in the discussion section.

Although from Figure 1 it appears that action can be taken on the hypothesis with N's of 1 or 2, the hypothesis, in this case, can not be rejected until at least 11 trials have been conducted and the odd sample correctly identified on all 11 trials. Consider the reject line, $D_o = 6.78 + 0.38\,N$, for sample sizes below 11, D_o, the number of odd samples correctly identified, is greater than N, the sample size; this points out the hypothesis can not be rejected until 11 trials have been conducted. Considering the accept line, $D_o = -5.28 + 0.38\,N$, for sample sizes below 14, D_o is negative. Thus the hypothesis can not be accepted until 14 trials have been conducted, and then only if none of the odd samples has been correctly identified.

Figure 1 is a convenient way of getting an overall view of the decision rule; its size as shown is too small for accurate use. If Figure 1 were drawn larger, say to a scale such that 10 inches on the abscissa represented an N of 100 and 20 inches on the ordinate represented a D_o of 100, it probably could be read with sufficient accuracy. Since the accept, continue, and reject regions are partitioned by straight lines, a graph of the decision rule is very simple to construct. This is why inequations 4 and 7 were not formulated in terms of proportions; such a formulation would have resulted in curves rather than straight lines.

The hypothesis testing decision rule can also be represented by a table. Table 1 shows the hypothesis testing decision rule for the same example, that is, a triangle test with the hypothesis $p'_1 = 0.33$, the alternate hypothesis $p'_2 = 0.43$, $\alpha = 0.05$, and $\beta = 0.10$. Such a table can be developed very rapidly with a desk calculator by considering inequations 4 and 7 as strict equalities and solving for D_o by substituting specific values of N.

In the triangle test example just completed, a one-sided alternate hypothesis with a one-tailed hypothesis test was appropriate. The sequential analysis approach for a two-sided alternate hypothesis with a two-tailed hypothesis test is different enough to be worthwhile considering. Suppose the relative preference between two products is to be investigated; the two products will be called K and L, which is, by the way, a combination of letters experimentally found to be relatively free from letter code bias *(4)*. Using a paired test, the null hypothesis in this case would state there is no preference between the two products; statistically the null hypothesis would be

$$p'_1 \neq 0.50.$$

Here p'_1 is the expected proportion of the total trials or paired presentations in which product K is preferred; p'_1 could have been defined as the preference proportion for product L instead of product K. The alternate hypothesis would appropriately state there is a preference between the two products; statistically the alternate hypothesis would be

$$p'_2 \neq 0.50.$$

In this case p'_2 is the expected proportion of the total trials or paired presentations in which product K is preferred. Thus p'_1 and p'_2 have the same

TABLE 1

Sequential sampling hypothesis testing decision rule for a triangle test where the hypothesis is $p'_1 = 0.33$, the alternate hypothesis is $p'_2 = 0.43$, $\alpha = 0.05$, and $\beta = 0.10$

N = Sample Size Accept if $D_o \leqslant A$ Reject if $D_o \geqslant R$

N	A	R	N	A	R	N	A	R	N	A	R	N	A	R	N	A	R
1	—	—	31	6	19	61	17	30	91	29	42	121	40	53	151	52	65
2	—	—	32	6	19	62	18	31	92	29	42	122	41	54	152	52	65
3	—	—	33	7	20	63	18	31	93	30	43	123	41	54	153	52	65
4	—	—	34	7	20	64	19	32	94	30	43	124	41	54	154	53	66
5	—	—	35	8	21	65	19	32	95	30	43	125	42	55	155	53	66
6	—	—	36	8	21	66	19	32	96	31	44	126	42	55	156	54	67
7	—	—	37	8	21	67	20	33	97	31	44	127	42	56	157	54	67
8	—	—	38	9	22	68	20	33	98	31	45	128	43	56	158	54	67
9	—	—	39	9	22	69	20	33	99	32	45	129	43	56	159	55	68
10	—	—	40	9	22	70	21	34	100	32	45	130	44	57	160	55	68
11	—	11	41	10	23	71	21	34	101	33	46	131	44	57	161	55	68
12	—	12	42	10	23	72	22	35	102	33	46	132	44	57	162	56	69
13	—	12	43	11	24	73	22	35	103	33	46	133	45	58	163	56	69
14	0	13	44	11	24	74	22	35	104	34	47	134	45	58	164	57	70
15	0	13	45	11	24	75	23	36	105	34	47	135	46	59	165	57	70
16	0	13	46	12	25	76	23	36	106	35	48	136	46	59	166	57	70
17	1	14	47	12	25	77	23	37	107	35	48	137	46	59	167	58	71
18	1	14	48	12	26	78	24	37	108	35	48	138	47	60	168	58	71
19	1	14	49	13	26	79	24	37	109	36	49	139	47	60	169	58	71
20	2	15	50	13	26	80	25	38	110	36	49	140	47	60	170	59	72
21	2	15	51	14	27	81	25	38	111	36	49	141	48	61	171	59	72
22	3	16	52	14	27	82	25	38	112	37	50	142	48	61	172	60	73
23	3	16	53	14	27	83	26	39	113	37	50	143	49	62	173	60	73
24	3	16	54	15	28	84	26	39	114	38	51	144	49	62	174	60	73
25	4	17	55	15	28	85	27	40	115	38	51	145	49	62	175	61	74
26	4	17	56	16	29	86	27	40	116	38	51	146	50	63	176	61	74
27	4	18	57	16	29	87	27	40	117	39	52	147	50	63	177	61	75
28	5	18	58	16	29	88	28	41	118	39	52	148	50	64	178	62	75
29	5	18	59	17	30	89	28	41	119	39	52	149	51	64	179	62	75
30	6	19	60	17	30	90	28	41	120	40	53	150	51	64	180	63	76

definition; the subscripts 1 and 2 designate expected proportions from the hypothesis and alternate hypothesis respectively.

The values chosen for α, β, and δ will be the same as the values used in the triangle test example: $\alpha = 0.05$, $\beta = 0.10$, and $\delta = 0.10$. The similarities and differences between the two examples will perhaps be more apparent this way. Using a $\delta = 0.10$ and recognizing there is inherent interest in preference proportions less than as well as more than 0.50, the alternate hypothesis has two specific values, $p'_2 = 0.60$ and $p'_2 = 0.40$, that will have to be considered.

To develop the sequential analysis rule in this case, it is necessary to calculate the constants for the accept and reject boundaries, inequations 1 and 5, respectively, twice: once for case I where $p'_1 = 0.50$, $p'_2 = 0.60$, $\alpha = 0.025$, and $\beta = 0.10$; and the second time for case II where $p'_1 = 0.50$, $p'_2 = 0.40$, $\alpha = 0.025$, and $\beta = 0.10$. The two cases arise because two specific values of the alternate are of interest. This two sided experimental interest explains the use of $\alpha = 0.025$; that is, $\alpha = 0.05$ has been split into two equal tail areas of 0.025 each. Equations 2, 6, and 3 are used in both cases for computing h_1, h_2, and S respectively.

For case I, $h_1 = 5.62$, $h_2 = 8.84$, and $S = 0.55$. Thus inequations 1 and 5, respectively, take the specific form of

$$D_o \leqslant -5.62 + 0.55\,N \text{ and} \tag{8}$$

$$D_o \geqslant 8.84 + 0.55\,N. \tag{9}$$

In the current context, D_o is the number of times product K is preferred out of the total of N paired trials or presentations. Inequation (8) defines the accept region; inequation (9) the reject region.

For case II, $h_1 = -5.62$, $h_2 = -8.84$, and $S = 0.45$; inequations 1 and 5 respectively take the specific form of

$$D_o \geqslant 5.62 + 0.45\,N \text{ and} \tag{10}$$

$$D_o \leqslant -8.84 + 0.45\,N. \tag{11}$$

Inequation 10 defines the accept region; inequation 11 the reject region. The directions of the inequality signs have been reversed in going from inequations 8 and 9 to inequations 10 and 11. Because inequations 8 and 9 apply to the case where the alternate hypothesis, $p'_2 = 0.60$, has a value greater than the value stated in the hypothesis, $p'_1 = 0.50$, the hypothesis will be accepted at the relatively lower values given by inequation 8 and rejected at the relatively higher values given by inequation 9. A parallel argument demonstrates the appropriateness of the directions of the inequality signs in inequations 10 and 11. The plus and minus signs on h_1 and h_2 change automatically in the computational procedure.

The hypothesis testing decision rule which inequations 8, 9, 10, and 11 represent is shown in Figure 2. The accept, continue, and reject regions are appropriately marked. The hypothesis can not be accepted until 114 pairs have been presented, and then only if product K has been preferred exactly 57 times. From 114 on, the accept region gets wider and wider; the mid-D_o of the accept region for any N over 114 is N/2 which is as expected with the hypothesis of $p'_1 = 0.50$. For N's less than 114, there is no meaningful accept region, for the accept region of case I is completely overlapped by the necessarily dominating continue and reject regions of case II, and the accept region of case II is completely overlapped by the necessarily dominating continue and reject regions of case I. In the situation being discussed, the accept-reject continue actions specified by the case I inequations and the case II inequations do not agree. Since case I is associated with the hypothesis of $p'_1 = 0.50$ and the alternate of $p'_2 = 0.60$, case I inequations dominate or take precedence in the upper half of Figure 2. Similarly case II dominates in the lower half of Figure 2. As the term half is used here, Figure 2 would be split in half by the line $D_o = N/2$. This argument also leads to the conclusion that it is impossible to accept the hypothesis below $N = 114$.

For sample sizes greater than 114, the continue region is composed of two parts; each part bounded by two parallel lines extending indefinitely. The fact that parallel lines never meet does not mean the experiment can or ever will continue indefinitely without a decision. This will be treated further in the discussion section.

There are two distinct reject regions. In both reject regions, the hypothesis, $p'_1 = 0.50$, is rejected; if the hypothesis is rejected because D_o

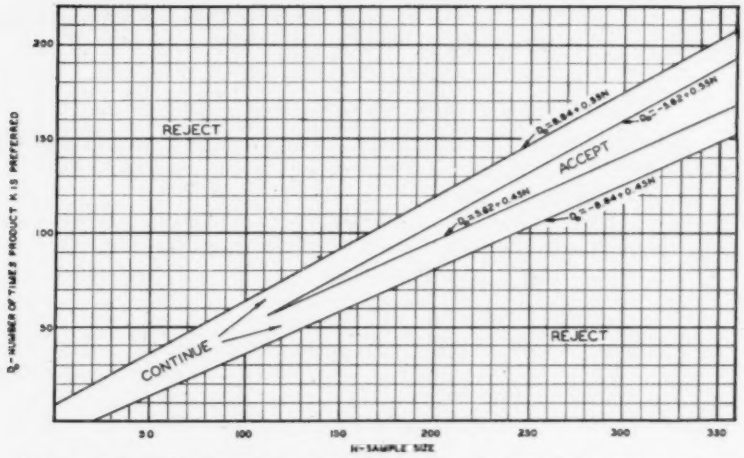


Figure 2. Sample size versus number of times product K is preferred for the hypothesis $p'_1 = 0.30$, the alternate $p'_2 = 0.60$ & $p'_2 = 0.40$, $\alpha = 0.05$, and $\beta = 0.10$.

is in the upper reject region, the alternate hypothesis, $p'_2 = 0.60$, is assumed to be true. If the hypothesis is rejected because D_o is in the lower reject region, the alternate hypothesis, $p'_2 = 0.40$, is assumed to be true.

Although, at first glance, it might appear that the hypothesis can be rejected for small sample sizes of say three or four, actually a sample size of 20 must be obtained before rejection is possible. To reject at 20 there must be perfect consistency in the results; that is, there must have been exactly 20 or exactly zero preference judgments for product K. For sample sizes below 20, the value of D_o required to reject the hypothesis is either greater than N or is negative as indicated by a check of Figure 2.

DISCUSSION

Underlying the use of sequential analysis for testing hypotheses associated with triangle, paired, and duo-trio tests is the assumption that the difference and preference proportions so obtained are binomially distributed. Sample proportions will be distributed binomially when obtained from a population where the event used in computing the proportion has a constant probability of occurrence. Since the triangle test null hypothesis stated there was no difference in the two products under investigation, the probability that the odd sample will be correctly identified has a constant value of $\frac{1}{3}$. The null hypothesis used with the paired preference test stated there was no preference between the two products; hence, the probability of one product being preferred over the other has a constant value of $\frac{1}{2}$. Similarly in the duo-trio test, the probability of identifying the standard sample has a constant value of $\frac{1}{2}$ since the duo-trio null hypothesis would state there was no difference between the standard product and the variant product under investigation. Thus the triangle, paired, and duo-trio test situations meet the assumption of a binomial distribution required in using the type of sequential analysis suggested in this paper.

It should be pointed out that in deriving inequations 1 and 5 and equations 2, 6, and 3 for h_1, h_2, and S, the exact binomial distribution is used. The method does not require a normal curve approximation to the binomial.

One distortion does occur in the derivation of the formulas. Burr *(2)* points out that the actual size of either α or β but not both could be a little higher than the value initially stated. For example, with stated values of 0.05 and 0.10 for α and β respectively, the actual α value could be as high but no higher than 0.055 while the actual β value would shrink correspondingly to 0.095 or less. It can be seen that this is not a serious problem in practical experimental work.

Abraham Wald is given credit for developing sequential analysis. His book *Sequential Analysis (9)* covers the theory and development of sequential analysis; Chapter 5 is particularly pertinent to the content of this paper as is Section 2 of *Sequential Analysis of Statistical Data: Applications (8)*. Both of these sources, although very worthwhile for the interested reader, cover sequential analysis primarily from the sampling inspection standpoint.

The main advantage of sequential analysis is that a decision regarding the hypothesis can be made with a relatively small sample size. The fixed sample size for a triangle test with the same specifications as used in the triangle test example in this paper would be 198 *(5)*. It is apparent from Figure 1 that decisions can be made before 198 trials have been conducted. The rule shown in Figure 1 will demand maximum sample sizes when sampling from a population where the probability of correctly identifying an odd sample is about 0.38; the value 0.38 in this example is mid-way between the hypothesis and the alternate hypothesis. Using the formula given by Burr *(2)*, the average sequential sample size would be 152 if p' were in fact 0.38. The average sample sizes would be 94 and 112 if p' were in fact 0.33 and 0.43 respectively and 14 and 11 for a p' of 0 and 1.00, respectively. So on the average the sequential approach, compared to the fixed sample size approach, allows a comparable decision to be made with a smaller sample size, drastically smaller as the true p' approaches 0 and 1.

Although the sequential approach has the inherent advantage of smaller sample sizes, the fixed sample size approach has a definite place. Certainly the experimenter in some instances has to plan ahead in terms of the exact sample size required; perhaps a fixed sample size approach is in order when experimental subjects have to be scheduled far in advance with little or no chance of changing the schedule, or when experimental foods have to be prepared or grown in advance in exact inflexible quantities. Such situations call for the fixed sample size approach.

The concern that the sequential analysis might drag on forever in the continue region has no basis in fact. According to Burr (*2*, p. 95), "This has been proved to have a probability equal to zero. Thus the plan is sure to terminate."

Others criticize sequential analysis as being too difficult. One has only to try it to find it far easier than it appears. In summary then, to use sequential analysis the accept and reject regions are respectively determined by

$$D_o \leqslant -h_1 + SN \text{ and} \tag{1}$$

$$D_o \geqslant h_2 + SN \tag{5}$$

The continue region is formed by the difference between the two lines associated with the above inequations. The constants h_1, h_2, and S are determined by appropriately substituting into equations 2, 6, and 3; these equations have identical denominators making the computational work easier than it might at first appear.

The experimenter, whose experiment is suitable for sequential analysis and who uses it, will be testing hypotheses at known α and β levels with minimum sample sizes.

LITERATURE CITED

1. Baker, G. A., Amerine, M. A., and Roessler, E. B. Errors of the second kind in organoleptic difference testing. *Food Research*, **19**, 206, 1954.
2. Burr, I. W. Fundamental principles of sequential analysis. *Ind. Qual. Cont.*, **IX** (6), 92, 1953.
3. Davies, O. L. *Design and Analysis of Industrial Experiments*. 1954. Hafner Publishing Company, New York, N. Y.
4. Ishler, N. H., Laue, E. A., and Janisch, A. J. Reliability of taste testing and consumer testing methods: code bias in consumer testing. *Food Technol.*, **8**, 389, 1954.
5. Radkins, A. P. Some statistical considerations in organoleptic research: triangle, paired, duo-trio tests. *Food Research*, **22**, 259, 1957.
6. Roessler, E. B., Baker, G. A., and Amerine, M. A. One-tailed and two-tailed tests in organoleptic comparisons. *Food Research*, **21**, 117, 1956.
7. Roessler, E. B., Warren, J., and Guymon, J. F. Significance in the triangle taste tests. *Food Research*, **13**, 503, 1948.
8. Statistical Research Group, Columbia University, *Sequential Analysis of Statistical Data: Applications*. 1945. Columbia University Press, New York, N. Y.
9. Wald, A. *Sequential Analysis*. 1947. Wiley and Sons, New York, N. Y.

SOME FUNGI THAT MIGHT CAUSE SOFTENING OF STORAGE OLIVES[a]

GEORGE D. BALATSOURAS AND REESE H. VAUGHN
Department of Food Technology, University of California, Davis, California

(Manuscript received June 8, 1957)

To insure adequate yearly production of canned ripe olives, the major product of the California industry, the bulk of the harvest must be stored in brines from a minimum of about 30 days up to 9 months or even longer[b]. During the first weeks the olives undergo a spontaneous fermentation usually predominated by lactic acid bacteria. Then, because it is exposed to the air, the surface of the brine becomes more or less covered with an aerobic, microbial film depending upon the location of the storage tanks. Indoors, the film may become as much as 2 inches thick whereas, if the brine is exposed to sunlight, it is restricted to islands of growth which develop in shaded areas.

Control of film formation has been haphazard. The heavy film that develops indoors generally is removed at intervals by skimming. Other methods of control have been found to be impractical or uneconomical for one reason or another. The islands of growth that develop on the brines stored out of doors generally have been ignored. However, olives stored outside undergo the same types of deteriorative softening as those stored indoors. Furthermore, the bulk of the olives are stored in the open.

The films contain a mixture of bacteria, molds and yeasts. Those formed indoors usually contain a high percentage of yeasts whereas the tough, leathery islands of growth formed out of doors consist largely of mold mycelium with some bacteria and yeasts interspersed therein. Bacteria isolated from the surface of olive storage brines are known to produce pectinolytic enzymes *(7)*. It is reasonable to believe that the molds and some of the yeasts isolated from these films also may produce pectinolytic enzymes *(17)*. Therefore, a study was made of the mold-like organisms isolated from the surface growths of olive brines stored out of doors. This report characterizes the kinds that were found.

EXPERIMENTAL METHODS

Source of cultures. The fungi were isolated from composite samples of the mycelial growths taken from the surfaces of brines stored out of doors. The tough, leathery, islands of growth were observed to vary in size, shape, and color. Those that floated free on the surface were roughly circular and varied in size from 1 to 2 cm. up to 12 to 15 cm. in diameter. Others remained loosely attached to the shaded periphery of the tanks or the tank closure braces. These latter growths were only a few cm. wide but might extend unbroken along the whole length of the shaded area. The colors of the mycelial growths also varied. Some were predominately brown, gray, green, rose, or rust in color. Others were varigated. Those with rose or rust hues seemed to predominate.

Fifteen composite samples were collected under aseptic conditions from 75 different

[a] Presented in part at the 16th Annual Meeting in Saint Louis, Missouri, on June 12, 1956.

[b] The Ascolano and Manzanillo varieties as well as some strains of other varieties can be pickled without preliminary brine storage. However, the economic limitations of processing facilities seem to prevent pickling of the entire crop as it is harvested. Therefore, at present, storage in brine appears to be obligatory.

storage brines. Each composite represented 5 separate tanks. All of the samples were collected in December 1955 at 3 plants in the San Joaquin Valley of California.

Isolation and purification of cultures. Composite samples (each weighed about 200 g.) were comminuted under aseptic conditions in a laboratory blender and then inoculated with a sterile loop onto sodium polypectate gel medium acidified to pH 4.0 (16)*. An attempt was made to pick different morphological types of colonies of pectinolytic molds from each sample. The selected cultures were purified on potato infusion, glucose agar.

Two methods were used for purification: the simple dilution technique commonly used for isolation of single colonies of bacteria and yeasts; and successive plating of minute portions of growing mycelium from the periphery of the colony. The direct dilution method was satisfactory for the isolates that produced abundant conidia and for the yeast-like *Oospora* culture. The direct transfer of bits of mycelium was used with those cultures that produced abundant mycelium but few or no conidia.

Although yeasts presented no serious problem in the purification of the cultures, contamination by aerobic, sporeforming bacteria caused considerable difficulty, even when the potato infusion agar was adjusted to pH 3.5. However, by repeated plating and checking the young cultures with the phase microscope the bacilli finally were eliminated. Thirty-six pure cultures were obtained for study.

Identification of the cultures. An attempt was made to allocate each of the purified isolates to its proper genus and species. Generic identification was relatively simple after a careful study had been made of the gross morphology and fructification of each culture. However, separation of the cultures into species was complicated and in a few cases was considered to be impossible in the course of this general study.

Morphological investigations were facilitated by use of the slide culture technique described by Wickerham *(19)*. The standard medium used throughout the study was potato infusion, glucose agar. The medium was prepared from the infusion of 200 g. of boiled, sliced potatoes made to 1 liter so that the final mixture also contained 2% added glucose and 1.5% agar per liter of medium. Water agar (tap water plus 1.5% agar) was used in some cases to foster sporulation by cultures which produced luxuriant mycelia but no conidia on the potato medium. General references used to assist in differentiation of the cultures included Alexopoulos *(1)*, Bessey *(2)*, Gilman *(3)*, Martin *(6)*, Snyder and Hansen *(10, 11, 12)*, Raper and Thom *(9)*, Stevens *(13)*, Thom and Raper *(15)*, and Waksman *(18)*. Individual references dealing with specific problems are listed at pertinent places in the text.

Physiological characteristics of special interest in relation to the role of the fungi as potential agents of spoilage of brined olives also were investigated. These studies included gross cellulolytic and pectinolytic properties, ability to utilize lactic acid, pH tolerance, and resistance to salt.

RESULTS

Identification of the cultures. The majority of the cultures (23 out of 36 isolates) were allocated to the genus *Fusarium*. Eight others were found to be members of the genus *Penicillium*. The genera *Aspergillus, Oospora (Geotrichium), Paecilomyces, Streptomyces,* and *Verticillium* were represented by single isolates.

The cultures of *Fusarium* were allocated to the following species:

Fusarium gibosum	— 2 cultures
Fusarium lateritium	— 4 cultures
Fusarium moniliforme	— 5 cultures
Fusarium oxysporum	— 6 cultures
Fusarium roseum	— 4 cultures
Fusarium solani	— 2 cultures

All but four of the cultures had characteristics considered to be typical

* The difficulties experienced in adjusting this gel medium to low pH values prompted further investigations concerning preparation of the medium. The results are described in another paper *(16)*.

of the species to which they were allocated. These 4 cultures were very difficult to identify. Two of the cultures finally were grouped with the species *F. gibosum*. The 2 remaining isolates were found to be similar to *F. oxysporum*.

The 2 cultures identified as *F. gibosum* formed very scant, pasty, grayish colored mycelia on the potato infusion agar. The outstanding characteristics of the 2 strains was the abundance of intercalcated and terminal chlamydospores they produced. The macroconida, noticeably wider in the middle, with pointed, hyaline ends, were 3 to 4 and very rarely 5 septate. Microconidia were not observed. Both isolates finally allocated to the species *F. oxysporum* formed abundant to scant grayish, cottony mycelia on potato infusion agar. Considerable variation in gross macroscopic appearance of the growth of these two latter cultures was observed during the investigation. Macroconidia were formed in clusters, had 3 to 5 septa and were quite pointed at the ends. Microconidia were one or non-septate. Chlamydospores were always present. It is possible that these 4 cultures are intermediate between the 2 species because *F. gibosum* and *F. oxysporum* have characteristics which overlap.

The 8 cultures of *Penicillium* were allocated to 2 species:

Penicillium chrysogenum — 4 isolates
Penicillium notatum — 4 isolates

The 4 cultures of *P. chrysogenum* and 2 of the 4 isolates of *P. notatum* were quite typical of the 2 species as described by Raper and Thom *(9)*. The 2 remaining isolates finally allocated to *P. notatum* could not be distinguished macroscopically from the 2 typical cultures. However, there were microscopic differences that were noticeable when slide cultures were studied. For example, the penicillus consisted of many divergent branches which were found to vary considerably in length. All of them ended in a verticil of metulae bearing sterigmata with long chains of conidia. Branches with verticles of metulae and others with verticles of sterigmata might or might not be at the same level. It was believed that the two isolates might be representative of *P. viriscens* as described by Thom *(14)*. However, this species was not recognized by Raper and Thom *(9)*. Therefore, since the 2 cultures in question could not be distinguished from the typical isolates of *P. notatum* in macroscopic appearance they were placed with that species.

One additional penicillium-like fungus was finally identified as *Paecilomyces varioti* because it had typical characteristics of the species as given by Thom *(14)*.

Four other cultures were identified only as to genus. These included one isolate of *Aspergillus, Oospora, (Geotrichium), Streptomyces,* and *Verticillium,* respectively. These 4 cultures had characteristics typical of their individual genera but were not sufficiently typical as to be easily recognized as species. Therefore, in the absence of enough cultures to make an acceptable comparative study it was thought best to restrict allocation of these 4 isolates to their respective genera.

Some physiological characteristics of the cultures. Since it was unknown whether these organisms could cause damage to olives held in storage brines, it was desirable to investigate their tolerance to pH,

resistance to salt, their ability to decompose lactic acid and to degrade pectin and cellulose. Investigation of these characters would assist in the evaluation of their role as possible agents of spoilage.

pH tolerance. The pH tolerance of the various cultures was tested on potato infusion, glucose agar adjusted to different values with hydrochloric or lactic acid. Olive storage brines have pH values that range from as low as 3.5 up to about 4.5 when fermentations are complete. Therefore, as shown in Table 1, only a few cultures would be restricted in growth by the pH values found in the brines of storage olives. It also is interesting to note that under the conditions of these experiments the cultures grew best at pH values near neutrality.

Salt tolerance. Olives generally are stored in brines that vary between about 5% and 8% (grams NaCl per 100 ml. of brine). The final salt concentration used depends upon the variety — the Ascolano and Sevillano fruits are quite prone to salt shrivel at concentrations much above 5% whereas the Manzanillo and Mission olives are not so easily affected. To the authors' knowledge the salt tolerance of fungi capable of growth on olive brines had not been investigated. Therefore it was desirable to obtain such information.

The salt tolerance tests were made in potato infusion, dextrose broth with increasing amounts of salt. Each culture that developed surface growth was transferred to another tube of the medium containing more salt. The initial concentration of sodium chloride was 5%. This was increased, first by 1% and later by 2% in each successive subculture. It is well known that the inhibitory effect of salt is increased at low pH values. Therefore, the salt tolerance tests were run in duplicate. One set had an initial pH value of 7.0. The other set was adjusted to pH 3.5. Results of the salt tolerance

TABLE 1

Effect of pH on growth[1] of fungi from olive brines

Genus and species	Number of cultures	pH optimum[2]	pH range[3]
Fusarium gibosum	1		
Fusarium lateritium	4		
Fusarium moniliforme	5		
Fusarium oxysporum	5	7.0	3.3-7.0
Fusarium solani	2		
Oospora sp.	1		
Paecilomyces varioti	1		
Aspergillus sp.	1		
Penicillium chrysogenum	4	5.0-7.0	3.3-7.0
Penicillium notatum	4		
Fusarium gibosum	1		
Fusarium oxysporum	1		
Fusarium roseum	4	7.0	4.2-7.0
Verticillium sp.	1		
Streptomyces sp.	1	7.0	5.2-7.0

[1] The medium was potato infusion, glucose agar.
[2] The pH optimum was the value at which growth occurred most rapidly and abundantly.
[3] The pH range indicated the values between which growth occurred in this investigation. The lowest value tested was 3.3.

studies are shown in table 2. The salt tolerance of the cultures was found to vary considerably. However, all cultures were able to tolerate the amounts of salt (5% to 8%) commonly used for storage of olives and most of them grew well at concentrations considerably greater than those used in the industry. Furthermore, with few exceptions the pH had no striking effect on the salt tolerance of the isolates. Therefore, it is apparent that the combination of salt and acidity (pH) found in olive storage brines is not sufficient to prevent the growth of fungi when the brines are exposed to the air.

Utilization of lactic acid. The ability of the cultures to utilize lactic acid was tested in an inorganic salts medium with the acid as the only carbon source. The test medium was prepared by first making 750 ml. of mineral salts solution which contained 2.0 g. NH_4NO_3, 1.8 g. KH_2PO_4, 1.4 g. K_2HPO_4, and 1.5 g. $MgSO_4$. Each salt was dissolved separately in a portion of hot distilled water and then all were mixed. The $MgSO_4$ fraction was added last to minimize the troublesome $MgNH_4PO_4$ precipitate which formed when all of the salts were dissolved together. The salts solution was sterilized by Seitz filtration. Then 8 ml. of lactic acid (95%) were added to 242 ml. of distilled water and the mixture was adjusted to pH 5.5 with NaOH. The solution was distributed in 5 ml. portions in Erlenmeyer flasks and sterilized (15 p.s.i.g. for 15 minutes). Then 15 ml. of the sterile salts solution were added by aseptic transfer to each flask. The finished medium was inoculated with the different cultures and incubated at room temperature for one week.

Criteria for utilization of lactic acid were development of an aerobic mat of growth and a significant increase in the initial pH value of 5.2 (after sterilization and mixture of the two components) to 8.0 or more with the majority of the isolates.

Most of the cultures utilized lactic acid quite well in the mineral medium whereas the mineral salts solution without lactic acid (control) failed

TABLE 2

Salt tolerance of the fungi from olive brines

Genus and species	Number of cultures	Maximum salt tolerance, % [1]	
		pH 3.5	pH 7.0
Fusarium gibosum	2	12.5	16.0
Fusarium lateritium	4	16.0	20.0
Fusarium moniliforme	2	12.5	12.5
Fusarium moniliforme	3	20.0	20.0
Fusarium oxysporum	1	12.5	13.5
Fusarium oxysporum	2	12.5	16.0
Fusarium oxysporum	3	16.0	18.0
Fusarium roseum	4	20.0	20.0
Fusarium solani	2	16.0	20.0
Oospora sp.	1	8.0	8.0
Paecilomyces varioti	1	10.0	12.5
Penicillium chrysogenum	4	22.0	22.0
Penicillium notatum	4	22.0	22.0
Sterptomyces sp.	1	8.0	8.0
Verticillium sp.	1	5.0	7.0
Aspergillus sp.	1	24.0	Saturation

[1] Grams of NaCl per 100 ml of potato infusion, glucose broth.

to support appreciable growth of any of the isolates. A good correlation was found between the abundance of growth and the change in pH of the medium. For example, 2 cultures of *F. moniliforme* and the single strains of *Aspergillus* sp., *Paecilomyces varioti*, and *Verticillium* sp. did not grow well and so caused a shift in pH from 5.2 only up to 6.0. In contrast the final pH values observed with all of the other cultures ranged from 7.0 to 9.1, thus indicating much better utilization of lactic acid in the same period of time (7 days). The growth of the rest of the fusaria was especially profuse when compared with the other cultures.

Pectinolytic activity of the cultures. The pectinolytic properties of the cultures were investigated by two qualitative methods. The first test involved utilization of pectic acid (Wichmann) as the only carbon source in a mineral medium *(8)*. The second method determined the ability of the cultures to soften a polypectate (Exchange Orange Products Co.) gel *(16)*.

Utilization of pectic acid was determined in the mineral salts medium described above. To make this medium the same amounts of the salts were dissolved as described in 500 ml. of distilled water and filter sterilized. Then 10 g. of pectic acid were suspended in 500 ml. of distilled water and adjusted to pH 6.2 with NaOH to form the water soluble sodium pectate. This solution was distributed in 10 ml. portions in Erlenmeyer flasks and sterilized at 15 p.s.i.g. for 15 minutes. Then 10 ml. portions of the mineral solution were added to each flask of pectate liquor. The finished medium was inoculated with the various cultures and incubated at room temperature for one week.

All but three of the isolates grew luxuriantly in the inorganic salts medium with pectic acid as the only carbon source. These 3 cultures (2 isolates of *F. moniliforme* and the one unidentified *Aspergillus*) grew as poorly as they did in the lactic acid medium already described. Since they also grew poorly in a 2% glucose, mineral salts medium this either might be a manifestation of their inability to use inorganic nitrogen well or an indication of their need for accessory nutrients.

The sodium polypectate gel medium *(16)* adjusted to pH 4.0 also was used to detect pectinolytic activity by the cultures. With this medium, incubated at room temperature for 3 weeks, softening of the gel was observed with all of the cultures except the unidentified isolates of *Streptomyces* and *Verticillium*.

Therefore, based on these 2 qualitative methods, it is apparent that most, if not all, of the isolates studied here possess pectinolytic properties and may be associated with the softening of olives.

Cellulolytic activity of the cultures. Since the softening of olives might result from the combined effects of pectin and cellulose degradation, an attempt also was made to determine the cellulolytic ability of the olive brine cultures. For this purpose cellulose was used in the mineral salts basal medium both with and without washed agar.

In the first test a circle of filter paper was placed in a 50 ml. Erlenmeyer flask and sterilized for 45 minutes at 15 p.s.i.g. Then 15 ml. of sterile mineral solution (prepared as before but with the salts added to 1 liter of distilled water) were added aseptically to each plugged flask. In the second series

enough mineral salts for 1 liter of medium were dissolved in 500 ml. of distilled water and sterilized by filtration. Then 500 ml. of 4% agar (washed for 15 days with 2 to 3 changes of water per day) were autoclaved at 15 p.s.i.g. for 15 minutes. The salts and agar solutions were mixed and poured under aseptic conditions into sterile Petri dishes. Then after the agar had solidified a circle of sterile filter paper was placed on its surface. In the third medium 2% purified cellulose powder (the grade used in paper chromatography) was incorporated in the washed agar before sterilization to replace the disc of paper used in the second test.

After preparation the 3 media were inoculated with the test cultures and incubated for 15 days at room temperature. Controls without cellulose were used in each of the 3 tests for cellulolytic ability of the cultures. The criterion of cellulose degradation was the relative abundance of growth in the media with cellulose as compared with that of the controls.

Clear cut results which demonstrated the cellulolytic ability of the various cultures were obtained only with the mineral salts solution and filter paper as the carbon source. Nineteen of the 21 isolates of *Fusarium* grew well in this medium. Growth of the fusaria, first evident on the submerged paper, soon spread throughout the liquid to form an abundant surface mat comparable to that produced on the potato infusion, glucose broth. In comparison, the 2 cultures identified as *F. gibosum* and all of the other isolates except the unidentified *Aspergillus* failed to grow in the medium. The *Aspergillus* culture grew slightly and produced concentric zones of conidia without an appreciable mycelial felt.

The same 19 cultures of *Fusarium* also grew moderately to abundantly in the other 2 test media. The 2 *F. gibosum* isolates also failed to grow in either of the washed agar media. Most of the rest of the cultures grew poorly in one or both washed agar media. The *Aspergillus* isolate as well as the species *Paecilomyces varioti* and the 8 penicillia which did not grow in the mineral salts, filter paper medium produced concentric sporogenous zones without evident development of mycelial felts on the two washed agar media. The culture of *Oospora* did not grow on any of the cellulose media whereas cultures of *Verticillium* and *Streptomyces* grew well on the washed agar, filter paper medium but failed to grow on the cellulose powder medium.

It was concluded that the majority of the species of *Fusarium* produced cellulolytic enzymes whereas the cellulolytic activity of the other organisms studied by the present authors was nil, or at most, limited. This conclusion was borne out by an additional experiment in which olives in 6% salt brine at pH 4.5, sterilized by intermittent pasteurization, were inoculated with various cultures of *Fusarium* and *Penicillium* used in this study. As would be predicted on the basis of their known pectinolytic activity, all of the cultures tested softened the olives. In addition extensive tissue disintegration (liquefaction) was caused by the cultures of *Fusarium*. This tissue liquefaction was interpreted as cellulose decomposition. These conclusions agree in the main with results described earlier by Marsh, Bollenbacher, and Raper *(5)* for cellulose decomposition by a variety of fungi.

SUMMARY

A study was made of 35 molds and 1 actinomycete isolated from the surface growths of olive brines stored out of doors. The majority of the iso-

lates (23 out of 36) were allocated to the genus *Fusarium*. Eight others were identified as members of the genus *Penicillium*. The genera *Aspergillus, Oospora (Geotrichium), Paecilomyces, Streptomyces,* and *Verticillium* were represented by single cultures. Of the fusaria, there were two isolates that belonged to the species *Fusarium gibosum*; 4 cultures identified as *F. lateritium*; 5 as cultures of *F. moniliforme*; 6 as isolates of *F. oxysporum*; 4 as *F. roseum;* and 2 cultures representative of the species *F. solani*. The cultures of *Penicillium* included 4 that belonged to the species *Penicillium chrysogenum* and 4 that were representative of *P. notatum*. One additional isolate was identified as *Paecilomyces varioti*. Four other cultures were identified only as to genus. These included 1 isolate of *Aspergillus, Oospora (Geotrichium), Streptomyces,* and *Verticillium,* respectively.

Since it was unknown whether these organisms could cause damage to olives held in storage brines their tolerance to pH, resistance to salt, ability to decompose lactic acid and to degrade pectin and cellulose were investigated. With the possible exception of the unidentified *Streptomyces* culture, it is likely that none of the cultures would be restricted in growth by the pH values of storage olive brines or the amount of salt contained in them. In addition, the cultures also utilized lactic acid as a source of carbon in an otherwise mineral medium. Furthermore, all but 2 of the isolates (*Streptomyces* sp. and *Verticillium* sp.) softened the polypectate gel medium. In addition cellulose was used as a carbon source by the fusaria but not by the other species. Therefore, it can be concluded that most, if not all of these organisms can be regarded as potential agents of spoilage of olives held in storage brines.

Acknowledgments

Support of this study from a grant-in-aid to the University of California by the California Olive Association is gratefully acknowledged.

Special thanks are due to Professors E. M. Mrak and W. H. English of the University of California at Davis for their assistance with the identification of some of the cultures.

LITERATURE CITED

1. Alexopoulos, C. J. *Introductory Mycology.* 1946. John Wiley and Sons, Inc., New York, N. Y.
2. Bessey, E. A. *Mycology and Taxonomy of Fungi.* 1950. The Blakiston Co., Philadelphia, Pa.
3. Gilman, J. C. *A Manual of Soil Fungi.* 1945. The Iowa State College Press, Ames, Ia.
4. Kertesz, Z. I. *The Pectic Substances.* 1951. Interscience Publishers, Inc., New York, N. Y.
5. Marsh, P. B., Bollenbacher, K., and Raper, K. B. The fungi concerned with fiber deterioration. II. Their ability to decompose cellulose. *Textile Research J.*, **19**, 462 (1949).
6. Martin, G . W. *Outline of the Fungi.* 1946. W. C. Brown Co., Dubuque, Ia.
7. Nortje, B. K. and Vaughn, R. H. The pectolytic activity of species of the genus *Bacillus*: Qualitative studies with *Bacillus subtilis* and *Bacillus pumilus* in relation to the softening of olives and pickles. *Food Research*, **18**, 57 (1953).
8. Phaff, H. J. The production of exocellular pectic enzymes by *Penicillium chrysogenum*. I. On the formation and adaptive nature of polygalacturonase and pectin esterase. *Arch. Biochem.*, **13**, 67 (1947).
9. Raper, K. B., and Thom, C. *A Manual of the Penicillia.* 1949. The Williams and Wilkins Co., Baltimore, Md.
10. Snyder, W. C., and Hansen, H. N. The species concept in *Fusarium*. *Am. J. Botany*, **27**, 64 (1940).

11. SNYDER, W. C., AND HANSEN, H. N. The species concept in *Fusarium* with reference to section *Martiella*. *Am. J. Botany*, **28**, 738 (1941).
12. SNYDER, W. C., AND HANSEN, H. N. The dual phenomenon and sex in *Hypomyces solani F. cucurbiteae*. *Am. J. Botany*, **30**, 419 (1943).
13. STEVENS, F. L. *Plant Disease Fungi*. 1950. Macmillan Co., New York, N. Y.
14. THOM, C. *The Penicillia*. 1930. The Williams and Wilkins Co., Baltimore, Md.
15. THOM, C., AND RAPER, K. B. *A Manual of the Aspergilli*. 1945. The Williams and Wilkins Co., Baltimore, Md.
16. VAUGHN, R. H., BALATSOURAS, G. D., YORK, G. K. II, AND NAGEL, C. W. Media for detection of pectinolytic microorganisms associated with softening of cucumbers, olives and other plant tissues. *Food Research*, **22**, 597 (1957).
17. VAUGHN, R. H., LEVINSON, J. H., NAGEL, C. W., AND KRUMPERMAN, P. H. Sources and types of aerobic microorganisms associated with softening of fermenting cucumbers. *Food Research*, **19**, 494 (1954).
18. WAKSMAN, S. A. *The Actinomycetes (Their Nature, Occurrence, Activities, and Importance)*. 1950. Chronica Botanica Co., Waltham, Mass.
19. WICKERHAM, L. J. Taxonomy of yeasts. U. S. Dept. Agr., *Tech. Bull.*, 1029 (1951).

NUTRITIVE VALUE STUDIES OF A WHEAT FLAKES, DRIED WHOLE MILK AND SUGAR MIXTURE

G. H. REUSSNER, JR. AND R. THIESSEN, JR.
Research Center, General Foods Corporation, Tarrytown, New York

(Manuscript received September 21, 1957)

Sherman and Campbell *(13, 14)* reported that a diet consisting of a mixture of one-sixth dried whole milk and five-sixths ground whole wheat supplemented only with table salt was adequate for the rat. When the proportion of milk powder was increased to one-third, however, there was a decrease in infant mortality and an increase in the average life span of adult rats. It was concluded that a mixture of the whole wheat, dried whole milk, and salt could give adequate nutrition to test rats, even when fed for numerous generations. Hart *(9)* has noted that as far back as 1918 it was known that mixtures of cereal and animal proteins gave efficient growth in animal feeding tests.

Rat feeding tests of breakfast cereals, such as the study reported by Sure *(16)*, indicate that "flaked" breakfast cereals which have undergone strong heat-processing are not adequate for animal growth. Since ready-to-eat cereals are usually consumed with milk, it would seem reasonable to assay these foods with the amount of milk usually consumed with them.

Studies with human subjects by Daum and Tuttle, *et al. (5, 6)* have shown that a basic cereal breakfast of fruit, cereal, milk, bread, and butter was nutritionally as favorable as an isocaloric bacon and egg breakfast for retention and utilization of nitrogen, phosphorus, calcium, iron, thiamine, and niacin. A 6-week rat feeding test by Beinert *(3)* indicated that a breakfast cereal, milk, and sugar mixture gave very good growth to test rats.

Using the per capita for the consumption of corn and wheat breakfast cereals in 1954 in the United States as reported by the U. S. Department of Agriculture *(17)*, it can be estimated that approximately 366 tons of these breakfast cereals were consumed. Along with the milk usually eaten with cereals, this is a considerable intake of protein. Therefore, it was felt that more information would be desirable on the role of cereal and milk combinations in nutrition.

It was the purpose of this study to determine the adequacy of a wheat flakes, powdered whole milk, and sugar mixture, with and without added nutrients, over an extended period of time.

MATERIALS AND METHODS

Preparation of diets. Basal diet (A) consisted of the proportions of 1 ounce of a pulverized wheat flakes breakfast cereal to 4 ounces of whole milk and 8 g. of sugar. Whole dry milk solids were used in place of fresh milk for convenience in the preparation of stable test diets. These ratios were chosen to represent the average portions of cereal, milk and sugar consumed in a cereal breakfast. A second diet (B) consisted of dried whole milk without supplements.

A third diet (C) was formulated to represent a "heavy" breakfast consisting of 2 slices of bacon, 2 eggs, 2 slices of toast with butter and 1 cup of soluble coffee with cream and sugar. The bacon was fried, frozen with powdered dry ice, and then pulverized. Dried whole eggs were used in place of fresh eggs and a dry cream substitute (Pream) was used in place of fresh cream for the coffee.

A second group of diets was prepared in which a complete vitamin and mineral supplement (diet E), a complete vitamin supplement (diet F) and a complete mineral supplement (diet G) were added to the basal cereal, milk and sugar diet. Calculations were made to determine what essential amino acids, vitamins and minerals might be deficient in the basal diet for the rat. From these data, a "complete" diet (H) was prepared containing the necessary supplements. A control diet (D) was made in which α-cellulose was added to the basal ration in place of the supplements. This non-nutritive cellulose was also added to the supplemental diets in such a manner that all rations contained equal amounts of the cereal, milk, and sugar. Composition of these diets is given in Table 1.

Animal assay methods. Each test diet was fed to 10 male weanling Carworth Farms Wistar rats weighing 50 to 70 g. Animals were housed in individual raised-bottom cages in an air-conditioned room kept at 75 ± 2° F. Food and distilled water were given *ad libitum*. Food consumption was determined every other day; the diets were kept under refrigeration. Fresh diets were made at monthly intervals. Weekly body weights were taken for the first 9 months of the test.

After 9 months on test 5 animals from each group were sacrificed by exsanguination after an overnight fast. At this time blood was collected from each animal for a determination of hemoglobin, red and white blood cell count, total protein and albumin. The heaviest, lightest and two medium weight rats from each group were selected for these studies. The carcasses of these animals were frozen in dry ice and pulverized after the livers were removed. The remaining animals were kept on test to determine the length of lifespan.

Analytical and physiological test methods. Blood serum protein determinations were done according to the method of Fister *(7)* in which 0.5ml. of the serum was treated with copper sulfate in the presence of alkaline sodium potassium tartrate to form the reddish-purple complex of the biuret reaction. The globulin was determined by subtracting the albumin from total protein values. The method for albumin was modified in that the globulin component was removed by filtration instead of centrifrigation. Hemoglobin determinations were made with the Sali hemoglobinometer.

Pooled blood from the animals which consumed the bacon and egg diet (C) and the basal cereal, milk and sugar diet (A) for 9 months was sent to the Institute of Medical Physics in Belmont, California, for serum β-lipoprotein determinations according to the

TABLE 1

Composition of test diets

Diet	Components							
	Wheat flakes	Whole milk solids	Sugar	Mineral mix [1]	Choline chloride	Vitamin mix [2]	α-Cellulose	Other
Group I								
A Basal — wheat flakes, milk and sugar	55.8%	28.4%	15.8%	——	——	——	——	——
B Milk (Parlac)	——	100.0%	——	——	——	——	——	——
C Bacon and eggs	——	——	6.9%	——	——	——	——	93.1%[3]
Group II								
D Basal — control	53.5%	27.2%	15.1%	——	——	——	4.2%	——
E Vitamins and minerals	53.5%	27.2%	15.1%	4.0%	0.1%	0.1%	——	——
F Vitamins	53.5%	27.2%	15.1%	——	0.1%	0.1%	4.0%	——
G Minerals	53.5%	27.2%	15.1%	4.0%	——	——	0.2%	——
H "Deficient" nutrients ..	53.5%	27.2%	15.1%	——	——	——	3.4%	0.8%[4]

[1] U.S.P. Salt Mix XIV

[2] Vitamins supplemented per kg. of diet: Biotin, 0.3 mg; 2 methyl-naphthoquinone, 2 mg; thiamine hydrochloride, 12 mg; pyridoxine hydrochloride, 20 mg; calcium panthenate, 50 mg; nicotinic acid, 90 mg; inositol, 200 mg; p-amino benzoic acid, 300 mg; riboflavin, 50 mg; DL-α-tocopherol diacetate, 100 mg; "Roche" dry vitamin A acetate with vitamin D_3 (325,000 units vitamin A/g. and 32,500 units vitamin D/g.) 12.3 mg.; crystalline vitamin B_{12}, 1 mg. Corn starch was used to dilute this mix to one gram.

[3] Includes 14.3% fried bacon, 21.8% dried whole eggs (June Dairy), 31.0% dried toast, 2.6% soluble coffee, 6.1% Pream (dried cream substitute), and 17.3% butter.

[4] Supplement contains 0.38% L-lysine hydrochloride, 0.25% DL-methionine, 0.1% DL-threonine, 0.1% L-histidine, 0.005% manganese sulfate and 0.01% DL-α-tocopherol diacetate.

method of Gofman *(8)*. In the procedure the ultracentrifuge is used to obtain the values for the S_f (0-12) and S_f (12-400) fractions.

Moistures were determined by drying the samples in the vacuum oven for 16 hours at 70°C. Fat determinations were made on the oven-dried livers and carcasses by a 6-hour extraction with ethyl ether on the Goldfisch apparatus. Nitrogen determinations were carried out by the Kjeldahl-Wilfarth-Gunning method according to the Association of Official Agricultural Chemists *(2)*.

Histopathology. Sections of liver tissue from the animals which were sacrificed at 9 months were prepared for histological study by staining with hematoxylin and eosin. These tissues were examined by qualified pathologists.

RESULTS

Body weight gain and food utilization. Body weight gains are shown in Table 2 for both 3 months and 9 months of the test period. Body weight gains from the basal wheat flakes and milk diet (A) were significantly greater than those by the whole powdered milk diet at 9 months ($P = < 0.05$), but the differences at 3 months were not significant ($P = > 0.05$). The bacon and eggs diet did not give significantly different body weight gains over those from the wheat flakes and milk combination at either 3 or 9 months.

After 9 months on test the basal wheat flakes and milk diet gave significantly better protein efficiency utilization than the milk ($P = < 0.001$) or bacon and eggs ($P = < 0.01$). On the other hand, the wheat flakes and milk resulted in inferior food efficiency than either the milk ($P = < 0.05$) or bacon and eggs ($P = < 0.01$).

In the group II series the ration containing the complete vitamin and mineral supplement gave significantly better body weight gain over that from the control cereal and milk diet after 9 months ($P = < 0.05$). At 3 months the body weight gain from the wheat flakes and milk diet containing the deficient nutrients was significantly greater than that obtained from the unsupplemented control cereal and milk diet ($P = < 0.01$). Otherwise, there were no significant differences in the values of body weight gains, food efficiency, or protein efficiency.

Liver data. After 9 months of feeding, the livers from half the animals on test were removed and weighed, before fat determinations were made. Liver data are given in Table 3. It was found that the liver fats of the animals consuming the bacon and egg diet were significantly greater than those consuming the basal cereal and milk diet ($P = < 0.01$). Liver fat contents calculated from the whole dried milk diet were not significantly different from those from the animals eating wheat flakes and milk ($P = > 0.05$).

In group II the average liver fats determined on animals consuming the mineral supplemented diet was significantly lower than that of the basal control diet ($P = < 0.05$). There were no other significant differences in liver fat in group II.

Histopathological studies indicated that some of the livers from rats consuming the bacon and eggs showed marked fatty infiltration. The livers from rats eating the milk diet appeared normal and showed no evidence of vacuole formation. Liver tissue from several of the animals consuming the basal wheat flakes and milk showed occasional cells with vacuoles within the cytoplasm which were suggestive of fat accumulation. Of the 5 livers studied, from the bacon and egg diet, 3 showed slight degrees of vacuolization within the cells greater than that seen in those from the basal cereal

TABLE 2

Average body weight gain and food utilization data [1]

Diet	Food consumed dry	Protein consumed	Body weight gain		Protein efficiency [2]	Food efficiency [3]
			Three months	Nine months		
Group I						
A W. Flakes, milk, sugar	3681 g.	456.3 g.	251.3±10.4 g.	381.5±17.1 g.[4]	0.833±0.019 [4]	0.103±0.002 [4]
B Milk (Parlac)	2983	751.8	228.5±11.4	323.3±11.8	0.431±0.014	0.112±0.003
C Bacon and eggs	2973	610.2	252.8±13.2	413.1±24.3	0.677±0.030	0.139±0.006
Group II						
D Basal control	3615	433.7	239.6± 8.0	361.0±12.3	0.835±0.020	0.100±0.007
E Vitamins and minerals	3871	464.5	264.2±11.2	412.3±12.7	0.851±0.016	0.104±0.002
F Vitamins	3745	449.3	251.6± 9.6	355.1±14.2	0.780±0.020	0.095±0.003
G Minerals	3598	431.9	244.7±10.3	364.2±12.4	0.851±0.020	0.101±0.005
H "Deficient" nutrients	3902	492.2	281.0± 8.3	398.1±14.9	0.808±0.025	0.102±0.003

[1] Compiled from 38 week (9 month) test data except body weight gains at 3 months. Average of 10 animals per group, except 9 in B and E groups.
[2] Protein efficiency calculated as grams gain in body weight per gram of protein consumed.
[3] Food efficiency calculated as grams gain in body weight for gram of dry food consumed.
[4] Standard deviation of mean.

and milk diet. The remaining 2 livers from the bacon and egg diet showed considerable numbers of cells which contained vacuoles, apparently due to fat. The livers from the bacon and egg diet were also large as is shown by the greater average liver weights of the animals given in Table 3.

Blood data. Values for serum protein, hemoglobin and blood cell counts in group II showed no significant differences. However, in group I it was shown that the rats consuming whole milk solids had significantly lower hemoglobin ($P = < 0.01$), red blood cell count ($P = < 0.01$), total serum protein ($P = < 0.01$), serum albumin ($P = < 0.05$) and serum globulin ($P = < 0.01$) when compared to the values obtained from the basal wheat flakes, milk and sugar diet. On the other hand, no significant differences were found for these values when the basal diet was compared to the bacon and egg diet.

Flotation rate measurements were made on 3 ml. of blood pooled from each of the 5 animals sacrificed from the basal cereal and milk diet and the bacon and egg diet. The Sf 0-12 for the bacon and eggs diet was 7 mg. % as compared to 48 mg. % for the cereal and milk diet. The bacon and egg diet resulted in a Sf 12-400 value of 105 mg. % whereas the cereal and milk gave a value of 0 mg.% for the corresponding fraction.

Carcass analyses. The protein, fat, moisture, calcium, phosphorus and ash contents of the carcasses of the animals in the groups fed the basal cereal and milk, the milk alone, and the bacon and eggs were determined. These values are listed in Table 4. The data show that on a per cent carcass basis the milk stored more protein, ash, and calcium and less fat than either the wheat flakes ration or the bacon and eggs. However, on a basis of actual amounts in the carcass there was less protein stored by the milk than by the other two diets.

The greater calcium storage on a per cent of carcass basis by the milk over that by the basal wheat flakes and milk diet was significant ($P = < 0.05$). The differences in per cent carcass composition of fat, protein, ash and phosphorus were not significant when the values from the wheat flakes,

TABLE 3
Summary of liver data

Diet	Liver fat [1]		Liver weight [2]	
	Dry basis	As is	As is	Per 100 g. body weight
Group I				
A Wheat flakes, milk and sugar	12.5%	3.6%	10.1g.	2.5g.
B Milk (Parlac)	15.1	4.3	9.8	2.7
C Bacon and eggs	29.8	10.6	15.1	3.3
Group II				
D Basal control	13.5	3.8	10.5	2.9
E Vitamins and minerals	12.2	3.4	10.1	2.6
F Vitamins	11.8	3.2	10.4	2.9
G Minerals	10.5	2.9	10.0	2.7
H "Deficient" nutrients	12.8	3.5	10.4	2.6

[1] Average of 5 animals per group except 4 on B and C and 3 on D diet. One liver from B diet and two from D diet were lost during determination.
[2] Average of 5 animals per group.

TABLE 4

Summary of carcass analysis data.

Diet	Protein [1]		Fat [1]		Moisture	Ash [1]	Calcium [1]		Phosphorus [1]	
A W. flakes, milk and sugar	41.9%	70.0g.[2]	48.9%	76.7g.[2]	53.1%	8.3%	2.4%	3.79g.[2]	1.4%	2.20g.[2]
B Milk (Parlac)	45.6	58.2	43.5	55.5	56.2	10.3	3.1	3.95	1.6	2.04
C Bacon and eggs	40.0	71.1	51.4	91.7	52.4	6.4	1.9	3.37	1.1	1.95

[1] Calculated on dry basis — average of 5 animals per group after nine months on test. Livers were removed before analysis was made.
[2] Average amounts stored in carcass.

milk and sugar diet were compared to those obtained from the milk or bacon and egg diets ($P = > 0.05$). The analysis of these 3 diets, including the calcium and phosphorus contents, is given in Table 5.

Longevity. Table 6 presents the survival data to date. It is shown that the various supplements in group II showed better survival than the unsup-

TABLE 5

Analysis of test diets [1]

Diet	Protein	Fat	Moisture	Calcium	Phosphorus	Ca/P ratio
A W. flakes, milk and sugar	12.6%	8.7%	3.2%	0.29%	0.43%	0.67%
B Milk (Parlac)	26.2	27.7	2.0	0.79	0.60	1.32
C Bacon and eggs	18.8	31.2	7.4	0.14	0.31	0.45

[1] All reported on an "as-is" basis.

plemented wheat flakes and milk, the bacon and eggs, and the whole milk solids diets. One animal died in the group on whole milk solids before the 9-month period. As a result, 4 rats were kept for longevity on this diet and 5

TABLE 6

Longevity Data

Diets	Number of animals surviving after the following periods [1]				Average survival time [2]
	18 months	24 months	30 months	36 months	
Group I					
A Wheat flakes, milk and sugar	4	3	1	0	614 days
B Milk (Parlac)	4	1	0	0	589
C Bacon and eggs	5	1	0	0	666
Group II					
D Basal Control	5	1	1	0	688
E Vitamins and minerals	5	4	2	0	856
F Vitamins	4	2	2	0	732
G Minerals	5	4	3	3	969
H "Deficient" nutrients	4	4	1	1	748

[1] At 9 months on test, four rats continued for a longevity evaluation on diet B and five on the remaining groups.
[2] Calculated by averaging the days survival (from birth until death), of all the animals kept for longevity in each group.

on all the other test diets. The complete mineral supplement (Salt Mix U.S.P. XIV) added to the basal wheat flakes and milk diet gave the longest life span.

DISCUSSION

Outstanding differences observed at the end of the 9 months of feeding were the low hemoglobin and serum protein values from the milk diet and the high liver fat from the bacon and egg ration. No supplement was added to the whole milk solids diet. The reason for this was that a comparison of the responses from the cereal and milk diet and the milk alone could show whether the addition of the wheat flakes increased the nutritive value of the milk. Since milk is poor in iron content, it might be expected that the animals in the group fed milk alone would become anemic.

It is interesting, even though the whole milk solids diet had a relatively high level of fat (28%) along with a poor mineral balance, that the livers from the rats on this diet appeared to be normal. On the other hand, the bacon and egg diet with a slightly higher fat content (31%), resulted in several enlarged, pale livers. The average liver fat from the bacon and eggs diet was twice as high as that from the milk diet (30% for the bacon and egg diet and 15% for the milk diet on a dry basis).

Bixby, Bosch, Elvehjem and Swanson *(4)* found that after 6 weeks of feeding, mineralized pasteurized milk gave an average liver fat of 7.1% as compared to 4.3% for animals on a stock ration. Powdered whole milk resulted in a liver fat of 4.9%. All these values were reported on a fresh liver basis. Spirling *et al. (15)* reported that rats consuming fresh pasteurized milk supplemented with trace elements and cod liver oil attained a mean life span of 563 days as compared to a mean life span of 638 days for a mixed stock dog food ration. A 10% dried whole egg supplement extended the life span from the milk diet to 720 days.

Flotation rate measurements showed that the blood from the rats consuming the bacon and egg diet in our study resulted in a much higher value in the Sf 12-400 class than the cereal and milk diet (105 mg.% as compared to 0 mg.%). From studies with human subjects Gofman, Tamplin and Strisower *(8)* suggested that "coronariness" may especially be related to high levels of lipoproteins of the Sf 12-400 class. However, in spite of the greater liver fat and higher Sf 12-400 values, the animals consuming bacon and eggs had as great a survival time as those consuming either unsupplemented wheat flakes, milk and sugar, or whole milk solids.

Meat and eggs are considered good sources of protein. The egg yolk of the bacon and egg diet is an excellent source of the lipotropic factor, choline. Wissler, Collins, Schroeder and Soules *(18)* found that a high fat diet containing lard along with high choline and low cholesterol resulted in lipomatous lesions of the coronary arteries in rats. Okey *(11)* reported that rats fed cholesterol supplements had enlarged, fatty livers which did not affect the survival rate adversely when compared to control rats without the cholesterol supplements.

The excellent body weight gains obtained from the basal wheat flakes, milk, and sugar diet indicates that the protein must be rather well-balanced since the protein level of the diet is only 12.5%. The body weights obtained by the basal cereal and milk diet in this test are in the range obtained in

other tests from stock dog food rations containing 25% protein. Another indication of a good amino acid balance of the basal diet is indicated by the relatively small increment in weight gain afforded by a supplement of lysine, methionine, threonine, and histidine. These amino acids were added at the levels calculated to give the amounts required by the growing rat as suggested by McCoy *(10)*. A more recent listing of amino acid requirements for the growing rat has been given by Almquist *(1)* and are presented in Table 7. Almquist has suggested a 0.5% requirement for threonine which is 0.1% lower than the value given by McCoy. Previous tests at our laboratories have indicated that microbiological analyses gave higher values for threonine in breakfast cereals than are obtained from assay by growth.

The levels of amino acids in the basal wheat flakes, milk and sugar diet were checked by microbiological analyses and are listed in Table 7. The phenylalanine content of the amino acid supplemented diet was slightly below the rat requirement, and it is possible that this amino acid may have limited the optimum utilization of the other amino acids. It must also be considered that microbiological amino acid analyses do not allow for losses in availability due to incomplete digestibility. Otherwise, the amino acid supplemented diet should have provided a good balance of amino acids.

One of the most rigorous tests of a diet is the ability of the animals to survive for extended periods without supplements. The 5 animals per group used in this longevity study were not sufficient to give a highly significant evaluation. However, this test does give the order of magnitude for life span which may be expected. A survival time of 3 years as attained by 3 out of 5 of the animals in group G is excellent for the rat. Most of the deaths on the test occurred with animals showing symptoms of respiratory disease. Of the animals surviving, no outstanding symptoms have been observed which seem to be related to any individual diet. Alopecia, respiratory disease, and sporadic lesions on the body and tail were noted in all groups.

It might not be expected that a mineral supplement would give better survival than a vitamin supplement to a basal wheat flakes and milk diet; however, the longevity data in Table 6 indicate that the mineral supple-

TABLE 7

Amino acid contents of wheat flakes, milk and sugar mixture as compared to rat requirements

	Basal mix [1]	Supplemented diet [2]	Requirement for young rat [3]
Arginine	0.55%	0.53%	0.2%
Histidine	0.30	0.39	0.4
Isoleucine	0.52	0.50	0.5
Leucine	1.40	1.34	0.8
Lysine	0.79	0.96	1.0
Methionine	0.19	0.43	0.4
Phenylalanine	0.62	0.59	0.7
Threonine	0.53	0.56	0.5
Tryptophan	0.18	0.17	0.2
Valine	0.70	0.67	0.7

[1] Diet A, basal wheat flakes, milk, and sugar.
[2] Diet H, with amino acid supplement. Values obtained by calculation based on microbiological amino acid analyses of the basal mix. The "D" form of threonine not included.
[3] Values suggested by Almquist *(1)*.

ment gave much better survival than vitamins. It is interesting that at 9 months the mineral supplemented diet also showed a significantly lower liver fat than the basal diet. Salt Mix, U.S.P. XIV, which was used for the mineral supplement contains Mn, Ca, Mg, K, Na, Cu, Fe, Al, I, Cl, and F. By calculation the Ca/P ratio of this diet was 1.1 as compared to a ratio of 0.7 for the basal cereal and milk diet.

Unknown factors in natural foods might also be related to the survival obtained in rat feeding studies. Schneider *(12)* postulated that there may be a chemical factor in natural foods which give extended survival to mice. Wheat and malted barley flour were suggested to be rich sources of this factor. These were the major raw products used in the production of wheat flakes. On a practical basis no one would consume during the life span only one diet such as bacon and eggs or cereal and milk; however, more data are being reported in the literature each year which give evidence that the total balance of nutrients in each meal may be more important than otherwise believed. There are many difficulties in translating data obtained from feeding tests with animals to those with human subjects. The information obtained in this study does emphasize that breakfast cereals and milk complement each other in such a manner that they may play a significant role in daily nutrition.

SUMMARY AND CONCLUSIONS

Diets containing *(1)* wheat flakes, whole dry milk solids and sugar, *(2)* whole milk solids and *(3)* bacon, eggs, toast, and coffee were fed to growing rats. At the end of 9 months some of the animals fed the bacon and egg diet had large, fatty livers and those consuming the milk alone had low blood protein and hemoglobin values. Rats consuming the wheat flakes, milk, and sugar mixture did not show such abnormalities.

The wheat flakes, milk solids, and sugar diet and the bacon and egg diet both gave good growth and survival to rats on long-term feeding. Longevity resulting from the wheat flakes and milk diet was extended more by a complete mineral supplement than by a complete vitamin supplement.

Acknowledgments

The authors are grateful to Mr. R. Bendon, Mr. R. Lind, and Mr. K. Schwartz for the care and feeding of the animals. We also wish to thank Mr. F. Germino for doing the clinical work.

REFERENCES

1. Almquist, H. J. The requirements for amino acids. In *Amino Acid Handbook*, ed. R. J. Block. 1956. Charles C. Thomas, Springfield, Illinois.
2. Association of Official Agricultural Chemists, *Official Methods of Analysis of the Association of Official Agricultural Chemists*, 7th ed. 1950. Washington, D. C.
3. Beinert, F. L. Growth on cereal and milk diet. *J. Home Econ.*, **44**, 119 (1952).
4. Bixby, J. N., Bosch, A. J., Elvehjem, C. A., and Swanson, A. M. Factors affecting the nutritive value of cows' milk. *J. Agr. Food Chem.*, **2**, 978 (1954).
5. Daum, K. W., Tuttle, W. W., Kisgen, R., Imig, C. J., Martin, C., Klapka, M. R., Sheehan, J. and Agle, M. Utilization of nitrogen, phosphorus, calcium and iron by men on different breakfasts. *J. Nutrition*, **27**, 475 (1951).
6. Daum, K., Tuttle, W. W., Kisgen, R., Imig, C. J., Bongey, R., and Martin, C. Effect of different types of breakfasts on niacin and thiamine excreted by men. *J. Am. Dietet. Assoc.*, **27**, 298 (1951).

7. FISTER, H. J. *Manual of Standard Procedures for Spectrophotometric Chemistry.* 1950. Standard Scientific Supply Corporation, New York, N. Y.
8. GOFFMAN, J. W., TAMPLIN, A., AND STRISOWER, B. Relation of fat and caloric intake to atherosclerosis. *J. Am. Dietet. Assoc.*, **30**, 317 (1954).
9. HART, E. B. Abuse of data on the biological value of proteins. *Nutrition Rev.* **10**, 129 (1952).
10. McCOY, R. H. Dietary requirements of the rat. In *The Rat in Laboratory Investigation.* ed. E. J. FARRIS AND J. Q. GRIFFITH. 1949. Lippincott Co., Philadelphia, Pennsylvania.
11. OKEY, R. Middle and old age in cholesterol-fed rats. *Proc. Soc. Exp. Biol. Med.*, **46**, 466 (1941).
12. SCHNEIDER, H. A. Nutritional and genetic factors in the natural resistance of mice to *Salmonella* infections. *Ann. N. Y. Acad. Sci.*, **66**, 337 (1956).
13. SHERMAN, H. C. AND CAMPBELL, H. L. Further experiments on the influence of food on longevity. *J. Nutrition*, **2**, 415 (1930).
14. SHERMAN, H. C. AND CAMPBELL, H. L. Nutritional well-being and length of life as influenced by different enrichments of an already adequate diet. *J. Nutrition*, **14**, 609 (1937).
15. SPERLING, G., LOVELACE, F., BARNES, L. L., SMITH, C. A. H., SAXTON, J. A., AND C. M. McCAY. Effect of long time feeding of whole milk to rats. *J. Nutrition*, **55**, 399 (1955).
16. SURE, BARNETT. Nutritional values of proteins in various cereal breakfast foods. *Food Research*, **16**, 161 (1951).
17. U. S. DEPARTMENT OF AGRICULTURE MARKETING SERVICE. NFS-76. *The National Food Situation.* (1956).
18. WISSLER, R. W., COLLINS, J. L., SCHROEDER, M., AND SOULES, K. Factors responsible for lipomatous and atheromatous lesious in coronary arteries of rat. *Federation Proc.*, **12**, 407 (1953).

SAMPLING METHOD FOR ANALYSIS OF COFFEE VOLATILES BY GAS CHROMATOGRAPHY

JOHN W. RHOADES
Southwest Research Institute
San Antonio, Texas

(Manuscript received November 27, 1957)

The aroma of coffee is of prime importance from the point of view of the consumer. The aroma not only produces a pleasing sensation by itself, but plays an important role in the acceptance of the brewed coffee. The flavor of brewed coffee is, in fact, a combination of the two senses, taste and smell. The aroma of coffee involves only the sense of smell and is, therefore, limited to the so-called volatiles in roasted coffee. Many of the compounds present in coffee aroma have been identified by various investigators *(2, 3, 5)*, but little information of a quantitative nature has been obtained, primarily due to inadequate methods of analysis. With the introduction of gas phase chromatography, a method of analysis for extremely small quantities of material has become available. The use of gas chromatography as a means of separation of the volatile constituents of onions and irradiated meat has been reported by Niegisch and Stahl *(4)* and Stahl *et al. (6)*. Dimick and Corse *(1)* used gas chromatography to separate the volatile constituents of strawberries. The quantitative measurement of a rather large number of coffee volatiles can be made in a comparatively short time by this technique and much information can be obtained as to the effects of roasting times and temperatures on different varieties of coffee, the nature of staling of coffee, storing and packaging of roasted coffee and the similarities and/or differences of regular and instant coffee. A sampling technique has been developed which produces characteristic and reproducible chromatograms of the volatiles obtained from ground roasted coffee. Since it is thought the sampling technique may be of help to others working on coffee production or research problems, the writing of this report does not await identification of all peaks obtained on the chromatograms. The method appears to be readily adaptable to flavor and odor problems of other food and beverage industries.

EXPERIMENTAL

Apparatus. The gas chromatographic instrument used was a Perkin-Elmer "Vapor Fractometer" Model 154 with an external gas sampling valve which is available as an accessory. Helium was used as the sweep gas at a rate of 100 cc. per minute. The column operating temperature was 45°C.

Since it is not the purpose of this report to discuss the theoretical aspects of gas chromatography, readers with an interest in this aspect are referred to A. I. M. Keulsmans, *Gas Chromatography* (Reinhold).

Preparation of column. The partitioning column used in this work consists of Johns-Mansville C22 fire brick as the solid support and Carbowax 1500 as the stationary liquid phase, in two meters of ¼ inch copper tubing.

The C22 fire brick was crushed and sieved to 40-60 mesh, water-washed, and then resieved. The dried brick was then coated with Carbowax 1500 in the ratio of 4 parts brick to 1 part Carbowax. Coating of the brick was accomplished by dissolving a predetermined amount of Carbowax in sufficient methyl alcohol to just completely wet the brick. The bulk of the alcohol was evaporated in a steam bath with constant stirring. When apparently dry, the coated brick was put in a glass tube which in turn was placed in an oven at approximately 80°C. A small flow of nitrogen was then passed through the coated brick overnight.

The two-meter copper tubing in a U-shape was filled with the coated brick and packed by use of a small vibrator, more coated brick being added as required. The open ends were tightly packed with about ½ inch of glass wool. It was necessary to bend the column in the shape of a double U so that it would fit in the column heating chamber of the "Fractometer."

Materials. All of the standard brands of coffee used in this study were vacuum packed drip-grind coffee purchased in local food stores. Coffee in pliable plastic containers was furnished by the Coffee Brewing Institute. Coffee samples which had been withdrawn from the roaster after various percentages of normal roasting time were supplied by the Duncan Coffee Company. Instant coffees used were locally well-known brands, packed in screw top jars. They were obtained from local food stores.

General sampling procedure. The sampling procedure in general consists of sweeping the volatile material from the heated, ground coffee by a stream of warm, moist helium. The helium and sample are passed through an ice water condenser to remove the bulk of the water. The coffee volatiles which pass through the ice water condenser are then collected in a liquid nitrogen trap. The collected sample at this stage consists largely of water and carbon dioxide. A preliminary column and trapping system is employed in such a manner that those materials which have an elution time greater than carbon dioxide, but less than water, can be recollected. A small amount of both carbon dioxide and water is also recollected but not in amounts sufficient to cause extreme "tailing" due to overloading of the column in the Fractometer as is the case with a sample which has not passed through the preliminary column. The recollected sample is vaporized and swept onto the partitioning column of the Fractometer and the chromatogram recorded.

It is necessary to determine the elution times of the carbon dioxide and water from the preliminary column so that the times of recollection can be determined. This was accomplished by connecting the preliminary column to the detector of the Fractometer and obtaining the chromatogram of the coffee sample while employing the same conditions as when recollecting the sample. The optimum recollecting time can then be determined from the chromatogram and used in all subsequent runs.

An internal standard has been added as an aid in obtaining the quantitative results. The chromatogram area of each unknown material is compared to the area of a known amount of standard for each analysis. The method of using an internal standard gives increased quantitative accuracy and, in this instance, has provided a convenient means of reporting results. Toluene has been used as the internal standard since its elution time is sufficiently different from those of the coffee volatiles as to cause no interference.

Sampling apparatus. A schematic drawing of the sampling apparatus is shown in Figure 1. The dimensions of coffee sampler E are such that it can be placed in a two-liter beaker of boiling water to a depth sufficient to avoid water condensation at the top of E-3. A small carbowax column H, approximately 10 inches over-all in length, is employed for preliminary separation of carbon dioxide and water, both of which are collected in excessive amounts. The sweep helium flow is maintained constant by a glass capillary at B and a constant pressure of 10 pounds at tank A. Rotometers at C and M are used only as checks of the flow of helium. Stopcocks at D and G are used to divert the helium either through the coffee sampler or by-pass line O. Stopcocks at I, J, and L are necessitated by the changes required during the various steps of sample collection, evacuation of trap K, and introduction of sample into the Fractometer. The coffee sampler E consists of four parts: (1) bubble chamber for moistening helium; (2) trap for entrained liquid; (3) sample chamber; and (4) condensate collector. The ice water condenser F may or may not be an integral part of the coffee sampler. A silicone rubber seal at T has been provided for the introduction of toluene which is used as an internal standard. The coffee sampler is connected into the system by using butt joints and short pieces of tygon tubing. All connecting lines are 7 mm. glass tubing with the exception of the connections between the gas sampling valve N and the stopcocks J and L. These lines are ¼ inch copper tubing and connected to the stopcock arms by butt joints and tygon tubing.

Detailed sampling procedure. Stopcocks are positioned so that the helium flow passes through the by-pass line O, the short column H, the trap K, and exits to the atmosphere through rotometer M. The coffee sampler is connected into the system and circulation of ice water through condenser F started. Twenty-five milliliters of distilled water is placed in the bubbler tube E-1 and 12 g. of 20-40 mesh ground, roasted coffee placed in E-3. Five-hundredths of a milliliter of an O-xylene solution containing 1% toluene is

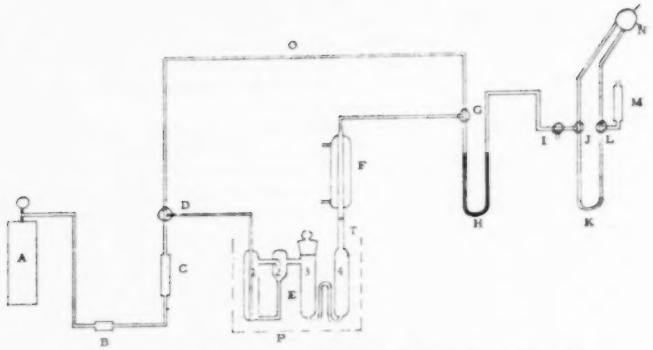


Figure 1. Schematic drawing of the sampling apparatus.

A — Helium Source
B — Flow Throttle
C — Flow Meter
D — Three-way Stopcock
E — Coffee Sampling Apparatus
F — Ice Water Condenser
G — Three-way Stopcock
H — Preliminary Column
I — Three-way Stopcock — Vent & Vac Line
J — Three-way Stopcock
K — Trap
L — Three-way Stopcock
M — Flow Meter
N — Sampling Valve
O — By-pass Helium Line
P — 2 Liter Beaker
T — Silicone Seal

injected into the system at T by use of a micro syringe. Column H is placed in a liquid nitrogen bath. The level of liquid nitrogen is above the column proper so that condensation of sample occurs in the glass wool packing which extends about one and one-half inches above the column packing. Stopcocks D and G are now positioned so that the helium passes through the coffee sampler. The entire coffee sampler, with the exception of the condenser, is placed in boiling water. The coffee volatiles are then collected for one hour. At the termination of sample collection the helium flow is again diverted through the by-pass line, and the coffee sampler removed from the system. The liquid nitrogen is removed from column H and is replaced by an empty Dewar flask. A stop watch is started at the time of removal of the liquid nitrogen from the column, and when sufficient time has elapsed for elution of the carbon dioxide as determined by previous experiments, trap K is immersed in liquid nitrogen so that the coffee volatiles may be recollected. After 4 minutes by the stop watch the empty Dewar is removed from column H and replaced by a Dewar containing water at 60°C. The water level should be above the level of the sample condensate to insure rapid vaporization. Recollection of sample in trap K is continued for a total of 13 minutes, by the stop watch, at which time the elution of xylene and water commences, again as determined by a previous experiment. The helium stream is momentarily diverted by stopcock G to exit to the atmosphere through the disconnected side arm tube. A vacuum line is connected to stopcock I and the liquid nitrogen raised on trap K so as to be above any condensate in the trap. Stopcocks I, J, and L are properly positioned and the trap K evacuated. Stopcocks J and L are now positioned so that the trap K is open to the lines connecting to valve N. The liquid nitrogen is removed from trap K and replaced by hot water to vaporize the collected sample. Rotation of the gas sampling valve N diverts the helium carrier gas flow of the Fractometer through the trap K and sweeps the collected sample onto the partitioning column. While the sample is being carried through the Fractometer, the preliminary column H is being swept free of water and xylene by proper positioning of stopcocks G and I, in preparation for the next sample.

Method of recording results. Only a few of the materials responsible for the various peaks obtained on the chromatograms have been identified. For purposes of reporting the results, the peaks have therefore been assigned numbers in order of their emergence from the column (see Table I). The area of each peak, including the toluene standard, was determined by obtaining the product of peak height by the peak width at half peak height. The weight of toluene which would produce an area equivalent to that determined for each peak was calculated by use of the formula:

$$W_x = \frac{A_x W_t}{A_t}$$

where:

A_x = area of peak in question
A_t = area of toluene peak
W_t = weight of toluene
W_x = weight of toluene which would produce area equal to A_x

The numerical value obtained has been termed the "toluene equivalent." This figure, when divided by the weight of coffee sample used, gives the "toluene equivalent" in micrograms per gram of sample as used in the table. The figures thus obtained are only of the correct order of magnitude as far as absolute values are concerned, but are of considerably more importance as a means of determining differences between the various samples.

Limitations. The method as outlined has two major limitations.

1. Only those materials which have an elution time less than water may be determined.
2. Separations are not complete in many cases, which impairs the accuracy of the area measurement.

The first limitation mentioned may not be too serious in this study, since the materials collected in the trap have the pleasant odor or aroma of coffee when vaporized. Incomplete separation of materials appears to be the limiting factor insofar as accuracy of measurements is concerned. Peak No. 13, which is methyl alcohol, exhibits poor reproducibility. This is to be expected in view of the fact that it forms an azeotrope with water. Other materials may also form azeotropes, but the effects appear to be minimized as indicated by the good reproducibility as compared to Peak No. 13. Collection of sample for the second hour shows little material other than methyl alcohol.

All of the work reported here was performed using the copper column. A stainless steel column has since been tried with no apparent differences in the recorded chromatograms.

RESULTS AND DISCUSSION

Preliminary experiments indicated that only a small portion of the coffee volatiles were removed from the ground coffee when dry helium was used as the sweep gas. The use of moistened helium was based on the principle of "displacement" chromatography whereby an absorbed material is "displaced" by a more strongly absorbed material. A comparison of the data in Table I shows the relatively large increase in collected coffee volatiles from duplicate coffee samples; dry helium being the sweep gas used for one sample, and moist helium the sweep gas used for the other sample. These data indicate a three- to five-fold increase in collected sample when moistened helium is used, and excludes the use of a dry sweep gas as a means of sample collection for quantitative purposes. Although no experiments have been performed on storage conditions of roasted coffee, the results here would indicate that the humidity of the storage conditions could have a marked effect on aroma retention.

A number of test series was performed and the chromatograms obtained of samples collected from ground coffee as described. The test series are tabulated below and the results of these tests recorded as the toluene equivalent in micrograms per gram of coffee sample in Table 2.

Series L data indicate that the analytical procedure is capable of detecting the differences in volatile components of various brands of vacuum packd coffee. These differences are probably due to the combined effects of variation in blends, roasting times, and possibly even packaging conditions.

Series Y data show a steady loss of volatile materials as would be expected of a coffee exposed to air, with the exception of Peak 15, which ap-

TABLE 1

Peak numbers toluene equivalents μg/gm

	1	2	3	4	5	6	7	8	9	11	13	14	15	16
Dry Helium Sweep Gas.	0.2	18.1	0.3	3.7	0.6	2.3	4.2	14.8	1.5	6.5	80.8	4.5	1.6	3.5
Moist Helium Sweep Gas	0.6	75.1	1.0	11.1	1.6	8.1	17.9	50.9	5.2	28.0	151.0	18.1	9.4	17.5

TABLE 2

Peak numbers toluene equivalent μg/gm

Series	Brand	Remarks	1	2	3	4	5	6	7	8	9	11	13	14	15	16
L	III		0.8	78.4	1.3	10.0	1.9	8.4	17.0	54.3	6.0	32.4	119.0	20.8	7.7	16.5
	I		0.5	57.0	0.5	8.3	1.5	5.6	18.0	37.5	3.7	28.8	122.0	13.5	—	13.2
	VI		0.6	47.0	1.7	12.7	2.2	4.5	12.4	52.8	5.1	20.2	103.0	14.1	4.3	12.3
	VII		0.8	49.2	0.5	6.0	4.1	9.8	13.3	113.0	20.2	39.7	78.0	19.9	—	7.2
	II		0.7	73.3	2.6	9.8	1.8	8.4	16.7	54.6	5.9	32.2	97.5	20.4	6.5	16.4
	VIII		0.5	42.4	—	9.4	2.0	5.1	8.9	*	6.6	19.4	97.6	14.4	4.1	8.8
	IV		0.4	38.7	—	3.6	1.7	4.1	11.2	39.0	4.3	18.0	74.6	9.8	—	8.9
	V		0.6	53.6	0.5	10.5	1.8	4.7	14.0	52.2	4.9	23.8	134.8	17.1	3.5	12.47
Y	II	0 hr	0.7	73.3	2.6	9.8	1.8	8.4	16.7	54.6	5.9	32.2	97.5	20.4	6.5	16.4
	II	45 hr	0.5	69.0	1.6	8.2	1.3	7.5	14.7	47.6	4.8	28.9	92.4	19.5	6.0	16.2
	II	168 hr	0.4	44.8	0.8	6.5	1.2	4.0	9.5	41.8	3.5	16.9	68.3	16.0	6.7	11.3
	II	195 hr	0.3	45.2	0.7	6.4	1.2	3.9	9.2	40.8	3.4	15.4	84.1	16.7	7.3	11.9
F	I	25%	—	5.9	1.2	—	—	0.2	1.1	1.7	0.3	2.6	17.6	—	—	—
	I	25%	—	5.8	1.1	—	—	0.2	1.1	1.6	0.4	2.2	*	1.9	—	—
	I	50%	0.6	8.4	1.0	0.1	—	0.3	3.5	3.1	—	6.3	44.0	1.2	1.8	—
	I	50%	0.6	8.5	1.0	0.1	—	0.2	3.4	3.1	—	6.1	46.8	0.9	1.2	—
	I	75%	0.4	21.5	1.2	7.1	1.2	2.7	11.2	28.9	3.0	16.9	72.3	5.7	2.9	6.2
	I	75%	0.4	20.8	1.2	6.7	1.2	2.6	11.2	28.4	2.8	16.3	68.8	5.4	2.6	6.8
	I	100%	0.6	43.4	1.1	10.4	1.5	5.0	10.7	42.1	4.4	19.8	88.6	12.6	4.5	11.0
	I	100%	0.6	44.6	1.0	10.4	1.5	5.0	11.4	42.2	4.4	19.0	82.6	12.1	4.6	10.4
	I	120%	0.7	38.2	0.9	10.2	2.3	5.6	10.3	*	7.1	22.1	70.8	11.6	7.7	8.7
	I	120%	0.7	36.9	0.9	10.1	2.2	5.5	9.6	*	6.6	21.2	88.4	12.4	7.8	9.5
J	X		—	*	—	0.2	0.3	1.7	2.0	9.7	1.0	5.7	7.1	13.0	—	3.9
	XI		—	*	—	3.9	0.3	4.2	16.6	*	1.8	24.2	35.9	15.1	5.2	10.5

* Off Scale

Series	Description
L	Various brands of vacuum packed drip grind coffee
Y	Coffee after exposure to air for various lengths of time. (Samples removed at intervals from single can).
F	Coffee samples which had been subjected to various percentages of normal roasting time
J	Two different brands of instant coffee

pears to have a slight increase with exposure. The increase is of about the same magnitude as the order of reproducibility and would therefore not be conclusive.

Series F data indicate a general increase in all volatile materials up to the 100% roast, after which there is a slight decrease in Peaks 2, 3, 7 and 16. Although these decreases are small, they are consistent in the duplicate determinations and would appear to be real decreases. The data on the duplicate determinations here show an order of reproducibility of about 10%.

Series J data show the differences between two brands of instant coffee. The samples from the instant coffees were obtained by bubbling helium through a water solution of the instant coffee and then collecting in the same manner as for the ground coffee. Since there is a difference in sampling procedure, these results may not be directly comparable to those obtained from ground coffee.

Data in Table 2 have been included in this report primarily to show the possibilities and sensitivity of the method. Although some information on the coffee volatiles has been obtained, there is as yet no correlation between the data and the aroma of coffee. A typical chromatogram of coffee volatiles is shown in Figure 2.

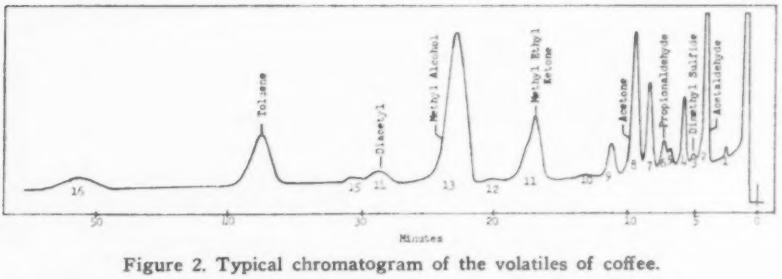


Figure 2. Typical chromatogram of the volatiles of coffee.

Infra-red identification has been attempted to a limited extent and resulted in positive identification of only the three major peaks. Tentative identification of a few other peaks has been obtained by coincidence of

elution times of a pure compound and a coffee volatile. Positive and tentative identifications are tabulated below.

Peak number	Positive identification	Tentative identification
2	Acetaldehyde	
3		Dimethyl sulfide
6		Propionaldehyde
8	Acetone	Methyl ethyl ketone
11		
13	Methyl alcohol	
14		Diacetyl

CONCLUSIONS

This preliminary investigation indicates that the analytical method will be of help in obtaining fundamental and practical information on roasted coffee, particularly where differences in analysis are of significance. Possible uses of the method or modifications thereof are:

1. Coffee roasting studies
2. Determination of differences in various varieties of roasted coffee
3. Staling studies
4. Study of packaging methods and materials
5. Instant coffee studies
6. Odor or aroma problems of other food products.

SUMMARY

A sampling procedure has been developed for the comparative analysis of the volatiles from ground roasted coffee by the use of gas chromatography. Chromatograms exhibiting some sixteen distinct peaks were obtained. Variation of peak areas with different coffee samples are distinctive and reproducible. Data have been included which show differences between coffee samples. These differences are far in excess of the apparent experimental error.

Acknowledgments

The work reported here has been sponsored by the Coffee Brewing Institute, Inc. of New York. The author wishes to thank Dr. Ernest E. Lockhart, Scientific Director of the Coffee Brewing Institute and Dr. Charles F. Raley for their assistance in this study, and Mr. Roger Brune and Mrs. Joan DiGirolomo for performing the many analyses required.

LITERATURE CITED

1. Dimick, K. P. and Corse, J. Gas chromatography — a new method for the separation and identification of volatile materials in foods. *Food Technol.*, **10**, 360 (1956).
2. Hughes, E. B., and Smith, R. F. The volatile constituents of roasted coffee. *J. Soc. Chem. Ind.*, **68**, 322 (1949).
3. Lockhart, E. E. The chemistry of coffee (Publication No. 25. The Coffee Brewing Institute).
4. Niegisch, W. D., and Stahl, W. H. The onion: gaseous emanation products. *Food Research*, **21**, 657 (1956).
5. Reichstein, T., and Staudinger, H. The aroma of coffee. *Coffee and Tea Inds.*, **78**, 91 (1955).
6. Stahl, W. H., Niegisch, W. D., Herk Jr., L. F., and Levy, E. J. The application of isolative gas-liquid partition chromatography and mass spectrometry to odor problems in food technology. Research Report, Analytical Chemistry, Series No. 5. Quartermaster Research and Development Center, Natick, Mass.

THE PIGMENTS OF OFF-COLOR COOKED TUNA MEAT[a]

W. DUANE BROWN, A. L. TAPPEL, AND H. S. OLCOTT
Department of Food Technology, University of California, Davis and Berkeley, California
and
Technological Section, Bureau of Commercial Fisheries, U. S. Fish and Wildlife Service Davis, California

(Manuscript received October 28, 1957)

The ordinary pink pigment which appears in precooked tuna and is maintained in the commercially canned product was recently characterized as a hemochrome *(1)*. With occasional fish or batches of fish, the normal pink color fails to develop during the precook and, instead, the cooked tuna meat takes on a tan to tannish-green color. Such fish, referred to in the industry as "green" tuna, are rejected. Since such fish are usually not recognizable in the raw state as being different from those which develop the normal pink color, "green" tuna represents a loss not only to the fisherman but also to the canners inasmuch as the fish have to be handled, cooked, and trimmed before the off-color is recognized.

The problem is general throughout the industry and is observed in Hawaii and Japan as well as in the United States *(5)*. Similar color problems occur in cured meat products *(2,6,7)*.

Results of studies of certain chemical properties of the pigments responsible for "green" tuna are presented in this report.

EXPERIMENTAL

Tuna. Samples of precooked "green" tuna loins were obtained from commercial tuna canneries in San Francisco, Terminal Island, and San Diego, California. Samples of precooked normal tuna loins were obtained from the San Francisco cannery. The species of tuna examined were yellowfin *(Neothunnus macropterus)*, albacore *(Germo alalunga)* and skipjack *(Katsuwonus pelamis)*. Samples of raw yellowfin loins were obtained through the courtesy of the Fishery Technological Laboratory, Seattle, from fish taken in the Gulf of Mexico. The exact treatment following catch of the latter fish was known; the samples were provided frozen in sealed half-pound cans.

Spectral measurements. Reflectance spectra were recorded with a Beckman DR spectrophotometer equipped with a reflectance attachment. Absorption spectra were recorded with the same instrument.

Reduction studies. Surface reduction of samples was achieved by sprinkling a solid reducing agent on the loin and spreading the material with a small brush. The commonly employed reducing agent was sodium hydrosulfite.

Heat treatments. The raw frozen samples described above were "precooked" in the laboratory in the unopened cans by heating at 100°C. for 45 min. Small amounts of tuna containing various additives were "precooked" by packing 10 g. of loin in clear glass vials (25 x 95 mm.), adding hot, freshly-boiled, distilled H_2O, capping and cooking at 10 p.s.i. for 15 min. Additions consisted of 0.01 and 0.1% sodium hydrosulfite or sodium ascorbate alone or in combination with 0.01 or 0.1% nicotinamide.

Oxidation-reduction potentials. Oxidation-reduction potential measurements of a 1:5 blend of tuna flesh in 0.1M phosphate buffer, pH 7.0, were made with a Beckman Model G pH meter, with platinum and saturated calomel electrodes. The material during meas-

[a] This research was supported by funds made available through the Saltonstall-Kennedy Act and administered by means of a collaborative agreement between the Fish and Wildlife Service and the University of California.

urement was contained in an air-tight plastic cup with inlet-outlet for purified N_2 gas which was bubbled through continuously.

Sulfhydryl group analysis. Sulfhydryl groups were determined on tuna flesh homogenates by the method of MacDonnell, *et al., (7)* by titrating with standard cysteine the excess *p*-chloromercuribenzoate remaining after a known quantity was allowed to react with sulfhydryl groups in the sample.

RESULTS AND DISCUSSION

General properties. Samples of off-color tuna seen in this study were a greenish-tan. There were slight variations among the samples, principally in the extent to which the green color was dominant; it was never intense. The color was essentially uniform throughout the thickness of the samples, and in no case was the desirable pinkness of normal tuna evident. A few of the samples smelled of hydrogen sulfide and an occasional rancid odor was detected. However, the odor was not consistent, either in the samples used in this study or in other samples observed in commercial tuna canneries.

Spectral studies. Since the normal pigments of tuna are hematin compounds *(1)*, off-color is obviously due to some alteration of their structure. The characteristic and distinct spectral properties of hematin compounds make it advantageous to use spectrophotometric methods for their study. Absorption spectra of the isolated pigments responsible for off-color were impossible to obtain since the pigments could not be satisfactorily extracted by a number of different methods. Consequently, the pigments were investigated by means of the reflectance spectra of the solid cooked tuna meat.

Reflectance spectra of 3 representative "green" tuna samples are shown in Figure 1. These are typical of spectra obtained on a total of 11 samples and show as much variation as was observed. Broad reflectance minima ap-

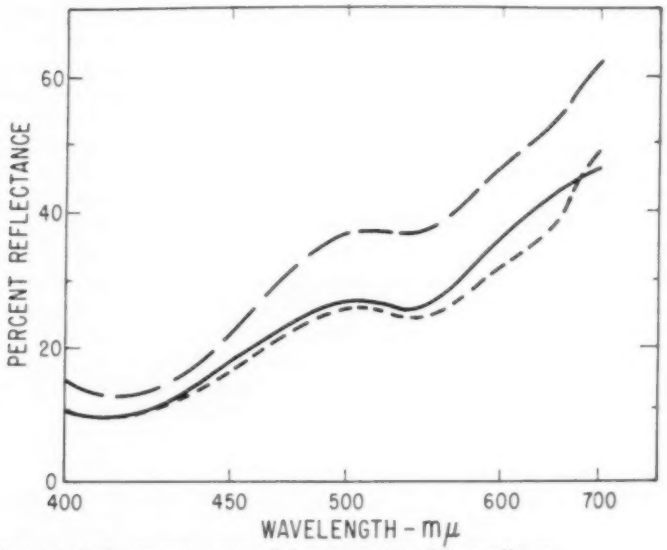


Figure 1. Reflectance spectra of three samples of "green" tuna.

pear at about 540 and 410-415 mμ; additionally there is a deflection in the spectral curve of some of the samples at about 650 mμ. In Figure 2 are shown

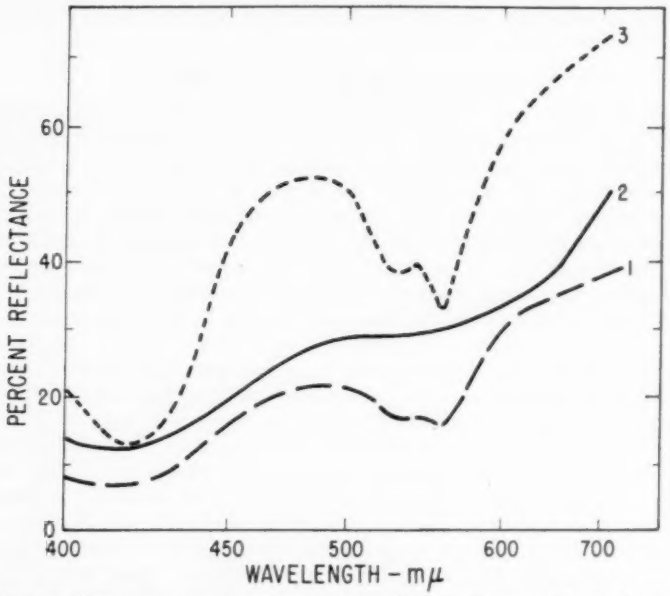


Figure 2. Reflectance spectra of a good tuna loin. 1. Untreated. 2. Following air oxidation. 3. Following sodium hydrosulfite reduction of the oxidized product.

reflectance spectra from a normal pink loin which was allowed to oxidize in air until it was no longer pink and was then reduced back to pink with sodium hydrosulfite. The spectrum of the pink loin with minima at 555, 530 and 415 mμ is that expected for the normal hemochrome pigment of tuna *(1)*. The spectrum of the oxidized loin is remarkably similar to those of the "green" tuna samples of Figure 1. Figure 3 shows reflectance spectra of a typical sample of "green" tuna untreated and treated with a reducing agent.

This spectral evidence leads to the conclusion that the off-color in the samples used in this study is due primarily to oxidation of the hemochrome pigments (Fe^{++}) to the hemichromes (Fe^{+++}). The typical reflectance spectra given by the "green" tuna samples (Figure 1) all include a broad minimum at 540 mμ, a characteristic of hemichromes *(3)*. Further, the air oxidation of a good tuna loin showing typical reflectance minima for the normal hemochrome led to the development of color in the loin which appeared identical to that observed in the "green" samples; the spectrum shown by this oxidized product is very similar to those of the off-color samples (Figure 2). Additional evidence is provided by the sodium hydrosulfite reduction of this oxidized pigment (minima 540 mμ) to the hemochrome (minima 555 and 530 mμ) (Figure 2) and the similar reduction of "green" tuna

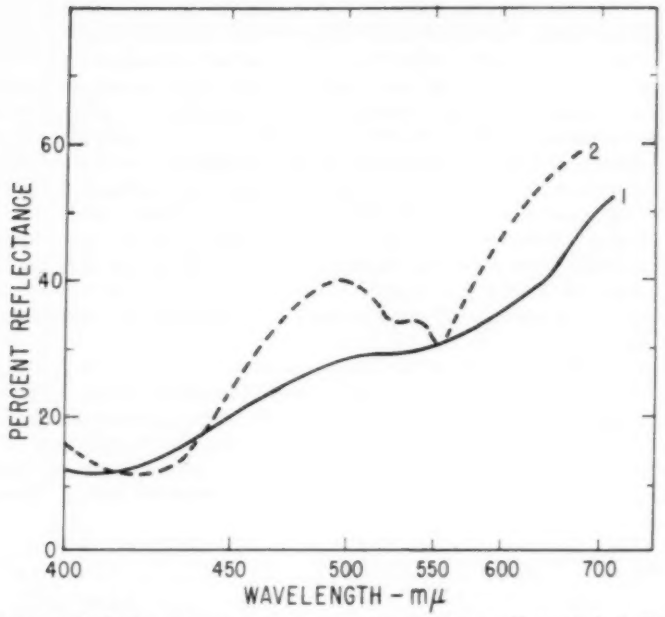


Figure 3. Reflectance spectra of a "green" tuna loin. 1. Untreated. 2. Reduced with sodium hydrosulfite.

showing the hemichrome spectrum to a pink material showing the hemochrome spectrum (Figure 3).

It is significant that the Soret absorption band at about 415 mμ appears, as a minimum, in all the reflectance spectra of "green" tuna observed. This band is a characteristic of the intact porphyrin ring structure of heme compounds *(3)*, hence its presence indicates that there has occurred no extensive oxidative destruction of the ring.

In an effort to put the insoluble pigment into solution, trypsin was used to digest a portion of "green" tuna loin. A measurement of the absorption spectrum of the final hydrosulfite-reduced clear filtrate was obtained. The spectrum showed absorption peaks at 555, 520 and 416 mμ, with the peak at 520 mμ slightly stronger than at 555 mμ. The spectrum thus appeared to be that of a hemichrome-hemochrome mixture. Care must be used in interpreting this result inasmuch as hemochrome and then hemichrome formation could have occurred during the digestion by a reaction of available heme with some nitrogenous product of the digestion procedure. However, it does indicate that no other pigment is present in the final extract; moreover, this approach may provide a useful tool for further studies.

Importance of reducing conditions. A total of some 25 samples of "green" tuna loins were treated with the reducing agent sodium hydrosulfite; in each case the pink color appeared following the reduction although there were differences in rate of appearance and intensity of pinkness. The sample

used for spectral measurements shown in Figure 3 is one of the "green" samples so treated. Good pink tuna loins allowed to air-oxidize to tan could be reduced in the same fashion to yield their original pink color. Other reducing agents, e.g. ascorbic acid, cysteine, will effect this reduction; however, they produce less pink color than the hydrosulfite.

This reduction to the hemochrome, noted in every case, strongly suggests that the off-color is due to hemichrome formation. Verdohemochromes could not be similarly reduced *(3)*. It is possible that there may have been small amounts of verdohemochromes formed; the resistance to reduction by any such compounds could be masked by the presence of the more abundant reducible hemichromes. In this connection it should be noted that the deflection in some of the spectral curves of "green" tuna at about 650 mμ might be caused by the presence of small amounts of verdohemochromes since they absorb in this region.

Figure 4 illustrates the molecular changes involved in the transformations of the hematin compounds described above. The structures are simplified, with the side chains being omitted; the circled nitrogens represent nitrogenous compounds coordinated with the iron atom to form the hemochrome.

Since the effect of reducing agents on "green" tuna is important in differentiating between hemichromes and verdohemochromes it was of interest to

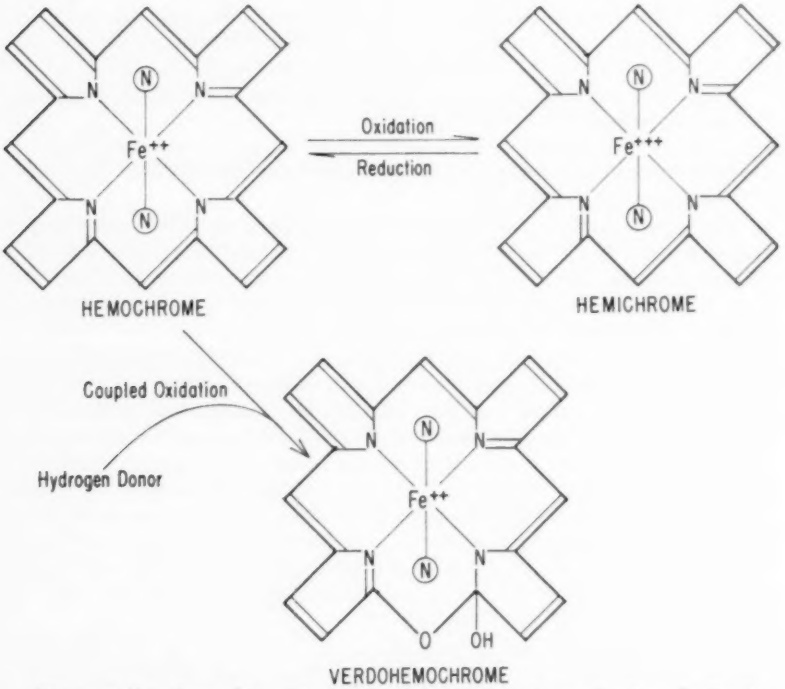


Figure 4. Reactions of hematin pigments involved in the production of off-color cooked tuna meat.

know the effect of added reducing agent on raw tuna known to develop off-colors on cooking. Three such samples of raw tuna were available; one of these gave particularly "green" tuna following cooking as judged by appearance and was quite resistant to reduction by sodium hydrosulfite. Additions of sodium hydrosulfite or sodium ascorbate in various combinations with nicotinamide, a known hemochrome-former, were made to small portions of the raw tuna sample and these were cooked. The only portions showing increased pinkness over the controls were those containing a relatively high level, 0.1%, of the stronger reducing agent, sodium hydrosulfite. Lower concentrations of this reducing agent were ineffective. This indicates that when strong reducing conditions are provided the desirable hemochrome formation will result even in samples known to yield very "green" tuna.

It appears that the problem relative to hemochrome-hemichrome formation is one of proper reducing conditions. It is not clear at this time whether the absence of the proper reducing circumstances is a function of the fish or of its handling, or a combination of these factors.

Relation of measured reducing capacity and treatment of fish to color. In preliminary studies attempts were made to correlate some measure of the naturally existing reducing capacity of a few available raw tuna samples with their known color-behavior. Included were loins known to show the desirable pinkness as well as those known to show off-colors. The raw samples used in the studies were from a small series of samples taken from yellowfin tuna which had been treated in various known ways following capture, i.e., stunned or allowed to thresh to death, bled or not, stored in sun or shade for several hours. No correlation was found between any of the several treatments or the size of the tuna, and the color of the precooked loins. Measurements of oxidation-reduction potentials of slurries of the raw loins were obtained; there was no correlation between such measured potentials and the color properties of the tuna loins. Also obtained were sulfhydryl group analyses of these various samples; again, no correlation was evident. Results of the studies appear in Table 1.

TABLE 1

Relation of reducing capacity and treatment to color

Sample	Weight of tuna, lbs.	Treatment after catch	Color	SH [1] groups	Redox [2] potential
Set A					
1	100-135	Not stunned, unbled, sun	Pink	0.94	0.055
2	100-135	Stunned, bled, shade	Slight pink	1.24	0.003
3	100-135	Not stunned, bled, shade	Slight green	1.40	0.090
4	100-135	Stunned, bled, sun	Green	0.90	0.070
Set B					
1	68-98	Stunned, bled, shade	Pink	—	—
2	68-98	Not stunned, bled, shade	Pink	—	—
3	68-98	Not stunned, unbled, sun	Slight pink	—	—
4	68-98	Stunned, bled, sun	Slight green	—	—

[1] Equivalent ml. 0.005 M cysteine/400 mg. wet wt. fish
[2] Millivolts, uncorrected for stn'd half-cell

Descriptions of the off-color problems of tuna given by various observers within the industry are widely divergent and sometimes conflicting. In this respect it should be emphasized that there may be more than one type of off-color. All of the samples used in this study behaved similarly and it appears that their off-color may have developed as a result of a single mechanism. Whether the proposed mechanism involved in producing "green" tuna, i.e. the oxidation of normal hemochromes to hemichromes, applies to other samples of "green" tuna can easily be tested by treating off-color loins with sodium hydrosulfite. If a pink color results, then at least part of the off-color is hemichrome in nature. A field survey designed to make such observations on large numbers of green tuna samples is planned. Additionally there is need for as much accurate detailed information concerning the history of individual cases of green tuna from catch (or before) through precook as can be obtained, in order to provide a lead to understanding the variability of reducing conditions in the flesh leading to the problem of hemichrome formation.

SUMMARY

Studies of the properties of the pigment responsible for off-color in so-called "green" tuna, including numerous reflectance spectra measurements, indicate that the pigment is a hemichrome. The hemichrome could in every case be transformed to the hemochrome by treatment with a suitable reducing agent. The hemichrome appears to result from the absence of proper reducing conditions. The overall relationships of off-color to the condition of the tuna and their subsequent handling could not be deduced.

Acknowledgments

The authors gratefully acknowledge the generous help of the many individuals in the various canneries and in the Fish and Wildlife Service who provided the samples used in this study. We particularly appreciate the help and advice given by Mr. Maurice E. Stansby, Chief, Pacific Coast and Alaska Technological Research, Fish and Wildlife Service, Seattle, Washington.

LITERATURE CITED

1. Brown, W. D., and Tappel, A. L. Identification of the pink pigment of canned tuna. *Food Research*, **22**, 214 (1957).
2. Jensen, L. B., *Microbiology of Meats*. 1954. The Garrard Press, Champaign, Ill.
3. Lemberg, R., and Legge, J. W. *Hematin Compounds and Bile Pigments*. 1949. Interscience Publishers, New York, N. Y.
4. Macdonnel, L. R., Silva, R. B. and Feeney, R. E. The sulfhydryl groups of ovalbumin. *Arch. Biochem. Biophys.*, **32**, 288 (1951).
5. Naughton, J. J., Frodyma, M. M. and Zeitlin, H. Nature of green or off-color condition in precooked yellowfin tuna. *U. S. Fish Wildlife Service Special Sci. Rep.* No. 197 (1956).
6. Watts, B. M. The retention of cured meat color. *Proc. Conf. on Research, Council on Research. Am. Meat Inst., Univ. Chicago, 9th Conf.* (1957).
7. Watts, B. M. Oxidative rancidity and discoloration in meat. *Advances in Food Research*, **5**, 1 (1954).

EFFECT OF COMPLEXING AND CHELATING AGENTS ON THE AFTER-COOKING DISCOLORATION OF POTATOES AND UPON THE IRON AND PHENOLIC CONTENT OF THE JUICE[a]

MERCEDES L. HUNSADER[b] AND FLORA HANNING
Department of Foods and Nutrition
School of Home Economics
University of Wisconsin
Madison, Wisconsin

The widespread occurrence of discoloration in potatoes after cooking has warranted study over a period of many years. The possible combination of iron and phenolic compounds was mentioned in the early work of Tinkler *(11)* and again more recently by Juul *(3)* and Mulder *(5)* as possible causative agents of the discoloring pigment.

A synthetic chelating agent, ethylene diamine tetra acetate or EDTA, was reported by Leonard and Stewart *(4)* to prevent iron chlorosis in citrus trees in 1953. Various sprays of EDTA were used on the Katahdin variety of potatoes by Smith and Muneta *(10)* who noted a decrease in the discoloration in such potatoes after cooking.

The plan of this study included attempts to modify the development of the pigment by spraying plants of a variety of potatoes, Ontario, known to discolor seriously after cooking and observing the effect on the color scores and on the phenol and iron content of juice from the light and dark areas, or by dipping the newly harvested tubers in solutions of chelates, sodium EDTA and sodium citrate, before placing them in cold storage and making the same observations as for the sprayed samples, or by evacuating slices of Ontario potatoes in solutions which might be expected to increase or decrease the phenol or iron content of the tissue.

EXPERIMENTAL

Ontario potatoes grown under controlled conditions were given certain plant spray treatments: iron sulfate, iron, sodium, magnesium, calcium ethylene diamine tetra acetate, EDTA, or sodium citrate. The design of the field plots included checks for the various treatments. Dip samples consisted of composites of control tubers placed in sodium citrate and sodium EDTA for ½ hour before being placed in cold storage. Halves of 24 or 27 potatoes of each treatment were cooked and the degree of after-cooking darkening was observed by 5 to 8 judges. The procedure for the cooking and scoring are reported in a paper by Hanning and Hunsader *(1)* and details of plant sprays by Hunsader *(2)*. After being scored, the potato halves from the 1955 treatments were carefully matched with the corresponding raw halves. The dark and light areas were cut from the cooked halves and similar areas were cut symetrically from the raw halves. Composites were made of dark and light areas of each spray and dip treatment and a single composite of the checks. Juice was pressed from the tissue and aliquots were taken for iron and phenol determinations.

Sixty-four potatoes from the check samples of the 1955 crop were divided into lots of 8 potatoes each. Each lot was peeled and cut into 2 mm. slices. Four of the lots were wrapped in aluminum foil and baked after evacuation. The slices were evacuated in 0.5% solutions of benzoic, boric, caffeic, chlorogenic, tannic, and citric acids; or of ethylene diamine tetra acetate or EDTA; or of iron sulfate. Two controls were used; (a) some slices were not given any treatment, and (b) some slices were evacuated in distilled water. Iron and phenol

[a] Publication approved by the Director of the Wisconsin Agricultural Experiment Station

[b] Present address: Kansas State College, Manhattan

determinations were carried out on the juice pressed from the slices as was done on the composites from the light and dark areas.

Variations of the procedures of Saywell and Cunningham *(7)* and Smith, McCurdy and Diehl *(9)* were used for the determination of iron. The color reagent was 4, 7-diphenyl-1, 10 phenanthroline and readings were made at 533 mμ on a Beckman DU Spectrophotometer. The phenol content was determined according to the procedure of Rosenblatt and Peluso *(6)* with tannic acid used as the standard. The color was read at 660 mμ on a Beckman DU Spectrophotometer.

RESULTS

The mean color scores in Table 1 show that none of the various sprays had

TABLE 1

The effect of certain plant sprays and tuber dips on the after-cooking discoloration of cooked potatoes

Year	1955		1956	
Sprays, dips and checks	Mean color scores [2]	Potatoes scored 7 or above	Mean color scores [2]	Potatoes scored 7 or above
		%		%
Iron EDTA [1]	5.23	11.1	5.05	12.5
Iron sulfate	5.47	14.8	5.74	25.0
Check	5.35	11.1	5.31	12.5
Sodium EDTA [1] weekly	4.21	7.4	5.58	16.7
Check	5.43	14.8	5.31	12.5
Sodium EDTA [1] early	4.76	7.4	—	—
Sodium EDTA [1] late	4.71	7.4	—	—
Sodium citrate	4.81	14.8	—	—
Check	5.16	7.4	—	—
Sodium EDTA [1] dip	5.51	14.8	—	—
Sodium citrate dip	5.79	25.9	—	—
Check, composite	5.31	11.1	—	—
Calcium EDTA [1]	—	—	4.77	0.0
Magnesium EDTA [1]	—	—	4.60	8.3
Check	—	—	5.31	12.5

[1] Salts of ethylene diamine tetra acetate.
[2] Maximum score = 10. Averages were based on 24 to 27 tubers, judged individually by 8 judges in 1955, and 5 in 1956.

a marked effect on the after-cooking discoloration of the tubers. Covering a period of 2 years, all of the tubers, whether treated or checks, were given low scores. The percentage of acceptable tubers of a score of 7 or above, ranged from 0 to 26%. Thus, it appears that the potato plant does not transport and use the spray materials in a sufficient amount to improve the color scores of the Ontario variety grown under these conditions. The effect of the dips on the discoloration in potatoes after cooking was little better than that of the sprays. However, of the 2 dips sodium citrate appeared to have more benefit than sodium EDTA. The effectiveness of the sprays on the Ontario variety of potatoes does not follow the pattern that was reported by Smith and Muneta *(10)* on Katahdins.

Iron and phenol determinations were done on juice pressed from the light and dark areas of cooked potatoes and on the juice from the corresponding areas of the raw halves. The data presented in Table 2 in terms of milli-

TABLE 2

The concentration of iron and of phenolic compounds in juice pressed from potato tissue

Spray and dip treatment	Phenol				Iron			
	Raw tissue		Cooked tissue		Raw tissue		Cooked tissue	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
	mg/ml	*mg/ml*	*mg/ml*	*mg/ml*	*mcg/ml*	*mcg/ml*	*mcg/ml*	*mcg/ml*
Sodium EDTA [1] weekly	.298	.328	.292	.384	1.8	2.1	2.6	5.9
Sodium EDTA [1] early	.282	—	.306	.384	0.9	1.8	2.7	6.0
Sodium EDTA [1] late	.280	.342	.316	.378	0.6	4.2	1.0	5.6
Sodium citrate	.292	.330	.316	.380	2.0	3.4	1.5	2.9
None	.278	.338	.310	.400	2.9	5.5	1.7	4.4
Iron sulfate	.286	.338	.300	.360	2.7	3.1	4.8	5.4
Iron EDTA [1]	.288	.328	.286	.376	2.1	3.7	1.9	5.8
Sodium EDTA [1] dip	.288	.334	.304	.364	4.2	6.1	3.5	3.3
Sodium citrate dip	.288	.330	.302	.362	4.1	4.2	2.6	3.3
None	.292	.328	.286	.386	4.7	6.8	3.4	4.8

[1] Salts of ethylene diamine tetra acetate.

grams of phenol and micrograms of iron per milliliter of juice, show that the juice pressed from the dark areas, whether raw or cooked, contained more phenol and more iron than the light areas. Apparently, cooking tends to release the phenolic components, since in both the light and dark areas the juice pressed from the cooked tissue usually contained more phenolic compounds than the raw. The late sodium EDTA spray had the most marked effect. It caused the concentration of iron in the dark areas in both raw and cooked tissue, but as noted above did not change the color score. The sodium citrate dip sample seemed to cause a mobilization of iron throughout the tissue as the difference between the iron reported in the light areas versus the iron in the dark areas was considered to be insignificant.

Table 3 gives the data for the concentration of phenols and iron in the juice from evacuated samples and expressed on the basis of 1 g. of original tissue. The effect of the various acid and salt solutions was compared with the distilled water treated slices. Water alone extracted phenols since the juice from the distilled water treatment contained more per gram than that from the untreated control. Three solutions had no effect on the amount of phenols released into the juice pressed from the raw tissue, boric, benzoic and caffeic acids; the others had some tendency to reduce the concentration of phenols in the juice. Baking caused a release of phenols into the juice except for the tissue treated with citric acid solution. Water rather than the solutes had a precipitating effect on the iron in the raw tissue, except for the iron sulfate and citric acid solutions, since the amount in the juice was significantly greater in those 2 treatments only. It may be noted that citric acid caused more iron to be present in the juice of raw tissue than any other solute except iron sulfate. Baking caused iron to be released into the juice from all tissue except for that treated with tannic or caffeic acid. Because a very large amount of iron was added to that sample as compared to the amount normally present in the tissue, large amounts were present in all lots so treated.

DISCUSSION

The list of solutes for the potato slices evacuated in solution included two,

TABLE 3

The iron and phenolic compounds of juice pressed from potato slices evacuated in various solutions.[1]

Treatment	Phenols				Iron			
	Raw tissue	Change from distilled water	Baked tissue	Change from distilled water	Raw tissue	Change from distilled water	Baked tissue	Change from distilled water
	mg/g	%	*mg/g*	%	*mcg/g*	%	*mcg/g*	%
None	.065	−52.6	.079	−40.1	0.85	41.7	1.05	−4.5
Distilled water	.137	—	.132	—	0.60	—	1.10	—
Benzoic acid	.138	0.5	.155	17.2	0.60	0.0	1.38	25.4
Boric acid	.136	−0.5	.144	9.1	0.58	−3.3	1.30	18.2
Caffeic acid	.138	0.5	.144	8.9	0.53	−11.7	1.08	−1.8
Chlorogenic acid	.115	−16.3	.164	24.4	0.60	0.0	1.65	50.0
EDTA[2]	.125	−8.4	.141	6.6	0.63	5.0	1.50	36.4
Iron sulfate	.118	−14.1	.135	2.7	5.60	833.3	8.05	631.8
Citric acid	.130	−5.3	.130	−1.1	0.95	58.3	1.25	13.3
Tannic acid	.124	−9.3	.139	5.5	0.65	8.3	0.60	−45.5

[1] Average of three or four determinations for each of four lots except for chlorogenic acid for which there were only two lots. In all cases, values are expressed in terms of grams of original tissue.
[2] Di-sodium ethylene diamine tetra acetate.

benzoic and boric acids, which are known to form complexes with phenols; three which are o-dihydric phenols, caffeic, chlorogenic and tannic acids, and which along with citric acid might be expected to form compounds with the iron in the potato tissue; and EDTA which is a chelating agent for iron and might be expected to solubilize it. Yet, there was meagre evidence of any of those influences in the raw tissue in the data of Table 3. The extraction of phenols and precipitation of iron by distilled water alone and the difference between raw and baked slices were the more marked variations. It may be that a weak tendency to form complexes or compounds was enhanced by heat in baking.

The problem of discoloration in potatoes after cooking appears to be one of metabolism. Citric acid is a cellular metabolite. Schwimmer *(8)* reviewed the enzyme systems of the white potato. He suggested that only one enzyme, polyphenolase, transfers the electrons from intermediates of the tri-carboxylic acid cycle to oxygen, and that if the transfer is interfered with in any way, the naturally occurring phenols are irreversibly oxidized with the formation of a dark pigment. Mulder *(5)* suggested that discoloration after cooking could be reduced in potatoes with the use of citric acid because it chelated iron so that it could not form ferric compounds on exposure to air. Our data on the iron content of potato juice lends some support to the theory of Mulder. However, with the addition of more citric acid by the dipping or spraying and especially by the tissue evacuation procedure, more citric acid was available to enter into the tri-carboxylic acid cycle and permitted a normal oxidation of carbon to take place, and a "normal" mobilization or utilization of the iron present in the tissue. Thus, with a normal metabolism possible the discoloring compounds may not be formed whether they be phenols, iron compounds, or an iron-phenol complex.

CONCLUSIONS

The use of a synthetic chelating agent, EDTA, as a plant spray or as a

tuber dip to reduce discoloration in potatoes after cooking did not prove successful in this study. Dark tissue appeared to release more iron and phenols in the juice than lighter colored tissue. Dilution with water and heat treatment as in baking had a marked influence on phenol and iron content of the tissues evacuated in various solutions.

Citric acid seems to play a prominent role in alleviating discoloration in potatoes after cooking. As a tuber dip, it caused more potatoes to be scored in the acceptable range; it caused a reduction of iron in the juice from the dark and light areas of tubers; it caused a release of iron into the juice from raw slices evacuated in its solution.

Acknowledgments

Appreciation is expressed to Dr. G. H. Rieman of the Genetic Department and to Mr. A. R. Albert of the Soils Department for their supervision of the growing of the potatoes.

LITERATURE CITED

1. Hanning, Flora, and Hunsader, Mercedes L., Problems involved in pretesting the tendency of potatoes to darken after cooking. *Am. Potato J.*, **34**, 347-358 (1957).
2. Hunsader, Mercedes L. A study of certain aspects of discoloration in potatoes after cooking. (Ph. D. thesis, University of Wisconsin, 1957).
3. Juul, F. Studier over kartoflens morkfarvning efter kogning. Julius Gjellerups Forlag. Kobenhavn (1949) Reviewed in *Am. Potato J.*, **27**, 32-35 (1950).
4. Leonard, C. D., and Stewart, I. An available source of iron for plants. *Proc. Am. Soc. Hort.*, **62**, 103-110 (1953).
5. Mulder, E. G. Mineral nutrition in relation to the biochemistry and physiology of potatoes. I. Effect of nitrogen, phosphate, magnesium, and copper nutrition on the tyrosine content and tyrosinase activity with particular reference to blackening of the tubers. Plant and Soil, **2**, 59-121 (1950).
6. Rosenblatt, M., and Peluso, J. V. Determination of tannins by photocolorimeter. *J. Assoc. Offic. Agr. Chemists*, **24**, 170-181 (1941).
7. Saywell, L. G., and Cunningham, B. B. Determination of iron. *Ind. Eng. Chem., Anal. Ed.*, **9**, 67-69 (1937).
8. Schwimmer, S. Enzyme systems of the white potato. *J. Agr. Food Chem.*, **1**, 1063-1069 (1953).
9. Smith, G. F., McCurdy, W. H. Jr., and Diehl, H. The colorimetric determination of iron in raw and treated municipal water supplies by use of 4:7-diphenyl-1:10-phenanthroline. *Analyst*, **77**, 418-422 (1952).
10. Smith, O., and Muneta, P. Potato quality. VIII. Effect of foliar applications of sequestering and chelating agents on after-cooking darkening. *Am. Potato J.*, **31**, 404-409 (1954).
11. Tinkler, C. K. The blackening of potatoes after cooking. *Biochem. J.*, **25**, 773-776 (1931).

ANTIOXIDANT AND COPPER BINDING PROPERTIES OF ONIONS[a]

EMMA JO LEWIS AND BETTY M. WATTS
Department of Food and Nutrition
The Florida State University, Tallahassee, Florida

(Manuscript received November 8, 1957)

Addition of antioxidants to fats and fatty foods has aided the food manufacturer to maintain a more acceptable product over a longer period of time. Many effective antioxidants, such as butylated hydroxyanisole, gallic acid, and nordihydroguaiaretic acid, come from nonfood sources. Antioxidants from natural foods have a distinct advantage, however, in that they are less subject to adverse Food and Drug Administration rulings.

The antioxidant properties of the natural spices have been described *(1, 4)*. The possibility that the onion, widely used for flavoring sauces and gravies, meat loaves, sausages, salad dressings, etc., might have antioxidant properties suggested the present study. Red and yellow onions contain as coloring pigments the flavonoid compounds anthocyanin and quercetin, respectively, whereas the white onion contains no flavonoid compound *(2)*. The flavonoid compounds have been studied as copper chelators *(5)* and as antioxidants *(10, 13)*. It may be that there is a connection between these activities and that the antioxidant activity is dependent on the ability of the compound to chelate copper or other heavy metal ions. Although these flavonoid pigments seem to be present in the skin of the onion, the juice from the meaty portion of the onion was of greatest interest since it is the juice which is ordinarily incorporated in food mixtures.

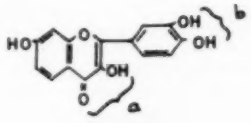
Previous investigations have shown the effects of ascorbic acid as an antioxidant either alone *(8)* or as a synergist with phenolic inhibitors *(3, 8)*. Ascorbic acid has been known to act as an antioxidant in dry fat systems, but it becomes a strong prooxidant in the presence of an aqueous phase *(12)*. Quite commonly, both a fat and an aqueous phase are present in foods. In meat there are sufficient traces of copper to cause oxidation of ascorbic acid making the free radical available, presumably, to attack the activated methylene group of an unsaturated fat. The compounds which synergize with ascorbic acid are those which complex copper *(9)*. It might be expected that the flavonoid compounds from onions might bind traces of copper and inhibit the catalytic affect of the metal ion on the decomposition of unsaturated fats, especially in the presence of ascorbic acid.

The object of the present study was to determine the conditions under which onions exhibit antioxidant properties, to study their possible synergism with ascorbic acid and to measure their copper complexing ability. Several other antioxidants were included in one or more of the experiments for comparison with the onion products. Garlic juice was tested for antioxidant activity because it is used like onions as a flavoring material. Liquid smoke[b] has previously been shown to synergize with ascorbic acid in meats as well as arti-

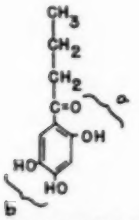
[a] Taken in part from a thesis submitted by Emma Jo Lewis in partial fulfillment of the requirements for the degree of Master of Science.

[b] Firmenich Company

ficial systems *(16)*. The 2,4,5-trihydroxybutyrophenone (THBP)*, a relatively new antioxidant, is of interest because its structure suggests that it might have excellent copper chelating as well as antioxidant properties. The structure of this compound and of quercetin, a flavonoid pigment of onions, are compared in Figure 1. Both compounds have active copper chelating sites (a) as well as orthodihydroxy groups (b) on the same molecule.



QUERCETIN



2,4,5 - TRIHYDROXYBUTYROPHENONE

Figure 1. Copper bindng sites (a and b) on some antioxidants investigated.

MATERIALS AND METHODS

Preparation of onion juice and onion extract. Onions were selected without regard to variety but with regard to color. Yellow onions were used unless otherwise indicated. The onion juice was prepared by chopping and blending the edible meaty portion and filtering the juice through a Schleicher and Schuell black ribbon filter paper. Samples of garlic juice were prepared in the same manner. Onion extracts were prepared by adding a given weight of the dried outer layers to 10 times the amount of boiling water. The mixture was stirred to remove air bubbles, allowed to steep for 10 minutes, and the extract was decanted off.

Antioxidant activity in artificial systems. Artificial aqueous fat systems previously described by Lehmann and Watts *(11)* were used to determine the presence or absence of antioxidant compounds. Controls lacking the test substance were included and all solutions were buffered at pH 5.8 with phosphate buffer at 0.02M final concentration. The aqueous and the fatty layers were added to their respective filter papers and the petri dishes sealed and incubated at 45° C. When the carotene in the fat phase was approximately half bleached, the samples were considered to be rancid. The various solutions tested for antioxidant activity were tested alone and in combination with ascorbic acid. All components of the tests were adjusted to pH 5.8. The liquid smoke and THBP were first dissolved in propylene glycol and diluted with water in the ratio 1:5:4, respectively.

Copper chelating ability. Following the procedure described by Frieden and Alles *(7)*, the Warburg apparatus was used to test for copper chelating ability. The principle of the method is as follows. The addition of cupric ions to ascorbic acid results in a rapid oxidation of the ascorbic acid with the production of dehydroascorbic acid and hydrogen peroxide *(6)*:

$$AH_2 + 2Cu^{++} \longrightarrow A + 2Cu^{+} + 2H^{+}$$

$$2Cu^{+} + 2H^{+} + O_2 \longrightarrow 2Cu^{++} + H_2O_2$$

* Eastman Company

The oxygen consumed during this reaction may be measured manometrically and under the conditions of the experiment, outlined below, amounts to approximately 115 microliters of oxygen. The addition of any compound capable of binding cupric ions inhibits this reaction. The degree of inhibition may be calculated from measurements of the total oxygen consumed by the ascorbic acid, copper mixture in the presence and absence of the test compound.

Solutions of the test substances were added to 5 X 10^{-6}M Cu solutions buffered at pH 5.8 with 0.01M phosphate buffer in the presence and absence of freshly prepared 0.008M ascorbic acid. The bath temperature used was 23° C. and air was the gas phase. Test substances were used at a 7-fold concentration in order to adjust to the larger concentration of ascorbic acid used by Frieden and Alles. Solutions of the tested compounds were prepared in the same manner as for the artificial fat systems. All solutions were placed in the main compartment of the Warburg vessel with the exception of the copper, which was added to the sidearm. Since carbon dioxide was not produced, alkali was omitted from the center well. Controls were included where necessary. Vessels were attached to their specific manometers and placed into the constant temperature bath. Five minutes were allowed for equilibration. Contents of the sidearm were tipped into the main compartment on all vessels before the stopcocks were closed and zero readings taken. Readings were taken at 5-minute intervals for a total time of 30 minutes.

Antioxidant activity in meats. Previous work by this laboratory *(14)* has demonstrated that rapid oxidation of some fatty component of meat tissues takes place when meat is heated. The oxidation is demonstrable by a large increase in TBA values and is accompanied by an undesirable flavor change. Antioxidant properties of onion extract and THBP, alone and with ascorbic acid, were therefore investigated in cooked ground beef.

Solutions of the test substances were incorporated into batches of the same lot of round of beef. Six variations were prepared: (1) a non-treated control, (2) ascorbic acid (0.03%), (3) onion extract (1%), (4) THBP (0.01%), and two variants of onion extract and THBP each with ascorbic acid. Each sample was well mixed and packed into No. 2 lacquered cans and sealed with a home canning sealer. Samples were heated in a steam kettle to an internal temperature of 70° C. *(14)*. Cans were removed from the bath and allowed to cool slightly. Cans were opened and the juice allowed to drain off. Samples were reground, well mixed, and stored at 6° C. TBA values were determined at intervals according to the method of Turner, *et al. (15)*.

RESULTS AND DISCUSSION

Antioxidant activity and copper chelation in artificial systems Cooked and uncooked samples of onion juice and of garlic juice at various concentrations were tested. Results are shown in Table 1. The antioxidant potency

TABLE 1

Antioxidant properties of onion and garlic juice

Sample	Final concentration in %	Time to turn rancid at 45° C. (days)
Control	....	11
Yellow onion, cooked	10	27
	90	122
uncooked	10	36
	90	> 122
Control	....	8
Garlic, cooked	1	13
	20	43
uncooked	1	15
	20	> 43

of the juices increased as the concentration of juice increased and the uncooked gave slightly better results than the cooked samples.

The meaty portion of the onion and the garlic bulb contains many compounds and the amount of flavones present was not known. Whether flavones were present in skin of the garlic has not been determined. Subsequent studies on the skin of the onion were made and are reported.

It was expected that enzymatic or acid liberation of polyphenols from glycoside combinations might enhance antioxidant effects of raw onion. Raw and cooked samples were allowed to stand several hours before filtering and incorporating into an aqueous fat system; however, such treatment caused darkening and decreased the antioxygenic effect of the juice. Similar treatment with onion extracts and raw onion pulp gave the same adverse results. Attempts to hydrolyze the extract with acid also failed to increase antioxidant potency.

It has been previously pointed out that the pro — or antioxidant properties of ascorbic acid can be determined by the presence of other factors in the medium. In the presence of strong copper complexing compounds the ascorbic acid becomes a fat antioxidant. Comparisons were therefore made of the antioxidant effect of various onion extracts, THBP and liquid smoke both in the presence and absence of ascorbic acid. Measurements of copper complexing were made on the same solutions. Results are shown in Table 2.

As might be expected the antioxidant potency of the yellow onion skin extract increased with increasing concentration up to the highest concentration tried, i.e., one gram of skin in 100 ml. water. The high protective factors obtained at this concentration as compared with those given by much higher concentrations of onion juice (Table 1) demonstrate that the antioxidants are far more concentrated in the skin than in the flesh of the onion. Extracts of red and white onion skins were progressively less potent than yellow.

Both yellow and red onion extracts gave 100% inhibition of copper catalysis of ascorbic acid under the conditions described; the white onion extract showed partial inhibition. All of these extracts inhibited to some extent the prooxidant effect of ascorbic acid on unsaturated fat, although none could be considered synergistic in the sense that the combination of ascorbic acid and onion extract was never more protective than the onion extract alone. It

TABLE 2

Antioxidant and copper binding properties of onion extracts and other compounds

Material	Concentration used in antioxidant experiments (in %)	Antioxidant Index [1]		Copper complexing ability
		No ascorbic acid	0.03% ascorbic acid	
Control	...	1.00	0.25	...
Propylene glycol	0.1	1.00	...	0
Yellow onion extract	0.1	1.20	...	...
	0.3	2.00	0.78	...
	0.6	4.00	2.10	...
	1.0	6.00	2.08	100
Red onion extract	1.0	5.00	3.00	100
White onion extract	1.0	2.16	1.67	84
Liquid smoke	0.02	3.20	3.80	0
THBP	0.1	7.35	9.65	100

[1] Calculated by dividing half bleaching time of variants by the half bleaching time of control.

must be remembered in interpreting these experiments that copper complexing ability is measured only for a period of half an hour, whereas the antioxidant experiments run over many days. It is probable that the compounds in onion skin responsible for copper chelation are destroyed by oxidation during the course of the antioxidant experiments.

The liquid smoke preparation differed from other antioxidants tested in that it showed synergism with ascorbic acid but no ability to complex copper. In fact, rapid uptake of oxygen occurred upon the addition of ascorbic acid to the smoke preparation, even without the addition of copper. In the absence of precise information on the composition of the smoke preparation, it is not possible to interpret these results.

THBP showed outstanding antioxidant ability by itself, synergized with ascorbic acid and gave complete inhibition of the copper catalysis.

Antioxidant activity in meats. Upon incorporating solutions of onion extracts and THBP in ground meat samples before cooking, several desirable effects were produced in the cooked stored meat. After one day's storage at refrigeration temperatures the control sample had lost its fresh cooked odor, but samples containing the onion extract and the THBP had retained it. Also the color of the onion extract and THBP samples remained somewhat pinkish rather than becoming the greyish color of the control sample. Until the onset of bacterial spoilage, the color of the samples remained as described above. Bacterial spoilage became evident after 8 days' storage and the experiment was therefore discontinued. At the last test period no samples containing either the onion juice extract or THBP, with or without ascorbic acid, showed an increase in TBA values over those obtained immediately after cooking. Results are shown in Table 3.

SUMMARY

The juice of onions and of garlic exhibits antioxidant properties. The extracts prepared from the onion skins have a more pronounced effect, especially that prepared from yellow onion skins. Onion skin extracts also exhibited copper chelating abilities and varying degrees of synergism with ascorbic acid. These activities are presumed to be due to flavonoid compounds. The 2,4,5-trihydroxybutyrophenone exhibited strong copper chelating activity, antioxidant properties and synergism.

Both THBP and extract of yellow onion skins were effective when incorporated in cooked meat both in the absence and the presence of ascorbic acid.

TABLE 3

TBA values of cooked meat samples containing antioxidants

Sample	TBA values after storage at 6° C. (in days)	
	0	8
Control	0.61	1.23
Ascorbic acid	0.65	1.28
Onion extract	0.61	0.63
Onion and ascorbate	0.66	0.58
THBP	0.49	0.46
THBP and ascorbate	0.59	0.48

LITERATURE CITED

1. Aggrawal, J. S. and Sethi, S. C. Stabilization of edible fats by condiments and spices. *Nature*, **166**, 518 (1950).
2. Bate-Smith, E. C. Flavonoid compounds in foods, *Advances in Food Research*, **5**, part 5, 1954, Academic Press Inc., New York.
3. Calkins, V. P. and Mattill, H. A. The kinetics of the antioxygenic synergism of quinones with ascorbic acid in fat systems. *J. Am. Chem. Soc.*, **66**, 239 (1944).
4. Chipault, J. R., Mizuno, G. R., Hawkins, J. M., and Lundberg, W. O. The antioxidant properties of natural spices. *Food Research*, **17**, 46 (1952).
5. Clark, W. G. and Geissman, T. A. Potentiation of effects of adrenalin by flavonoid (Vitamin P-like) compounds. *J. Pharmacol. Exptl. Therap.*, **95**, 363 (1949).
6. Flitman, R. and Frieden, E. Cuprous Ion Formation in Cupric Ion Catalyzed Oxidations. *J. Am. Chem. Soc.*, **79**, 5198 (1957).
7. Frieden, E. and Alles, J. Subtle interactions of cupric ion with nucleic acid and components. *J. Biol. Chem.*, **230**, 797 (1958).
8. Golumbic, C. and Mattill, H. A. Antioxidants and the autoxidation of fats. XIII. The antioxygenic action of ascorbic acid in association with tocopherols, hydroxyquinones, and related compounds. *J. Am. Chem. Soc.*, **63**, 1279 (1941).
9. Kelley, G. G. and Watts, B. M. Effect of copper chelating agents on the prooxidant activity of ascorbic acid with unsaturated fats. *Food Research*, **22**, 308 (1957).
10. Lea, C. H. and Swoboda, P. A. T. On the antioxidant activity of the flavonols, gossypetin, and quercetagetin. *Chemistry and Industry*, 1426 (1956).
11. Lehmann, B. T. and Watts, B. M. Antioxidants in aqueous fat systems. *J. Am. Oil Chem. Soc.*, **28**, 475 (1951).
12. Scarborough, D. A. and Watts, B. M. The prooxidant effect of ascorbic acid and cysteine in aqueous fat systems. *Food Technol.*, **3**, 152 (1949).
13. Spannuth, H. T., McGuine, T. H., and Crapple, G. A. Improvement of keeping quality of edible fats by some commercially available tannins. *Oil and Soap*, **23**, 110 (1946).
14. Tims, M. and Watts, B. M. Protection of cooked meats with phosphates. (In press).
15. Turner, E. W., Paynter, W. D., Montie, E. J., Bessert, M. W., Struck, G. M., and Olson, F. C. Use of the 2-thiobarbituric acid reagent to measure rancidity in frozen pork. *Food Technol.*, **8**, 326 (1954).
16. Watts, B. M. and Faulkner, M. Antioxidant effect of liquid smokes. *Food Technol.*, **8**, 158 (1954).

SUCCINOXIDASE ACTIVITY OF SOME ANIMAL TISSUES

A. L. TAPPEL AND RUTH MARTIN
Department of Food Technology, University of California
Davis, California

(Manuscript received September, 1957)

The succinoxidase system consisting of succinic dehydrogenase linked to the cytochrome electron transport system has been identified as the major surviving respiratory enzyme in meat *(1, 2)*. Succinoxidase and the other surviving respiratory enzymes in meats are responsible for the continuing consumption of oxygen and the resulting deoxygenation of the oxymyoglobin in the interior of the meat and on the surface whenever the meat is placed in an oxygen tight enclosure. Reduction of the oxygen concentration makes myoglobin more labile to oxidation to metmyoglobin. If on the other hand removal of oxygen in meat is desired, then the succinoxidase plus added succinate would serve as an efficient oxygen scavenger. For these and other important reasons it is desirable to know the succinoxidase activity of the commercially important meats. Information on the activity of succinoxidase activity in animal tissues is largely confined to assays on small laboratory animals; the work of Lawrie *(4)* on the succinoxidase activity of various horse muscles and Andrews *et al. (1)* on beef muscle are important exceptions.

EXPERIMENTAL

Succinoxidase assay. Animal tissues were ground in a glass homogenizer using 2 g. tissue per 10 ml. 0.25 M. sucrose following the method of Potter *(5)*. The homogenate was neutralized to pH 7.3 with 0.1 M sodium hydroxide. Succinoxidase activity was determined manometrically at 20° C. Three ml. of homogenate and 1 ml. 0.5 M sodium succinate pH 7.3 were added to each reaction vessel; the center well contained potassium hydroxide for absorption of carbon dioxide. Although it is known that added cytochrome c will increase the rate of oxygen absorption *(6)*, cytochrome c was not added because we wished to measure the "surviving" succinoxidase system. Controls contained the homogenate but no added succinate. Oxygen absorption was measured every 5 minutes for 1 hour and from the slope of the linear plots of oxygen absorption vs. time the succinoxidase activity was determined.

Samples of animal tissues. The 2-g. sample required for assay was obtained from the larger samples of animal tissue after they were ground cold in a meat grinder and thoroughly mixed.

Beef samples were obtained from 21 animals distributed as follows: 5 Brahford steers, 5 Brahford heifers, 6 Hereford steers and 5 Hereford heifers. These cattle were raised under controlled conditions and were slaughtered in an abattoir. Average live weight was 990 lbs. and average grade of the carcass was *choice*. After holding at 33° F. for an average of 10 days, samples of ¼ to ½ lbs. were taken from the sirloin tip.

Lamb samples were obtained from 3 female Suffolk-Corniedale lambs immediately after sacrifice. The animals were 2 years old and averaged 100 lbs. live weight. One lb. samples were obtained from the liver and the rib eye muscle of chops from the 11th to 13th rib of the rack and loin area.

Pork samples were obtained from 7 swine of various known breeds and included 4 barrows and 3 gilts of average age 6 months and average dressed weight of 170 lbs. One-half lb. samples of muscle, mainly the rib eye, were taken from loin chops from the 11th to 13th rib area. The chops were cut from pork loins obtained through commercial channels. Average time between slaughter and enzyme assay was 5 days.

Samples of chicken thigh and leg, breast and liver were taken immediately after sacrifice of 7 chickens representing various known cross breeds in which the sire and dame

were from New Hampshire dames. Average age was 7 weeks and average live weight was 2 lbs.

Mice samples included the muscles of the thigh and leg and the liver which were taken immediately after sacrifice of 8 females of Swiss-Webster breed. Average age was 8 weeks and average live weight was 22 g.

RESULTS AND DISCUSSION

The succinoxidase activity of the animal tissue is shown in Table 1. All of the control reactions containing homogenate and no added succinate gave very low values except for the livers which were 0.21, 0.76, 0.19 μl. O_2/hr./mg. wet tissue for lamb, chicken, and mouse liver, respectively. The negligible oxygen absorption for muscle homogenates in the absence of added succinate shows the absence of substrates for enzyme catalyzed oxidation. These results are similar to those of Andrews *et al. (1)* who also found lack of substrate for the surviving respiratory enzymes of muscle. This high oxygen absorption for liver homogenates without added succinate indicates the presence of some substrates for enzyme catalyzed oxidation.

In beef cattle, the largest group examined, there were no significant differences of succinoxidase activity between steers and heifers or between Brahfords and Herefords. Differences in the succinoxidase activity of the other species of animals were small but the number of samples did not allow an evaluation of the significance of the differences. Kunkel *et al. (3)* were able to relate cytochrome oxidase activity of some animal tissues to the three-fourths power of the body weight and hence to basal metabolic rate. In this study the same muscle areas are represented in the thigh and leg samples of the cattle, chicken and mice and in the loin samples of the lamb and sheep. However, detailed intercomparisons are not warranted because of the small numbers of species represented in each of these 2 groups. It is interesting, however, that the ratios of succinoxidase activities of the cattle and mice and of the swine and lamb are similar to the ratios of cytochrome oxidase activities of large and small animals found by Kunkel *et al. (3)*.

SUMMARY

The succinoxidase activity of some important cuts of meat from beef

TABLE 1

Succinoxidase activity of animal tissues

Animal	Location of sample	Succinoxidase Activity
		μl O_2/hr./mg. wet tissue ± standard deviation
Beef cattle	Sirloin tip	0.25 ± 0.06
Hog	Loin	0.20 ± 0.06
Lamb	Rack and loin	0.42 ± 0.04
	Liver	1.30 ± 0.06
Chicken	Thigh and leg	0.23 ± 0.05
	Breast	0.16 ± 0.04
	Liver	1.03 ± 0.14
Mouse	Thigh and leg	0.92 ± 0.20
	Liver	1.82 ± 0.30

cattle, hogs, lambs, and chickens was determined by manometric measurement of the oxidation of succinate added to a homogenate of the meat.

Acknowledgments

The authors wish to thank M. T. Clegg, Department of Animal Husbandry, U. K. Abbott, Department of Poultry Husbandry, and M. Simone, Department of Food Technology, for providing samples of animal tissues.

LITERATURE CITED

1. Andrews, M. M., Guthneck, B. T., McBride, B. H., and Schweigert, B. S. Stability of certain respiratory and glycolytic enzyme systems in animal tissues. *J. Biol. Chem.*, **194**, 715 (1952).
2. Grant, N. H. The respiratory enzymes of meat. I. Identification of the active enzymes. *Food Research*, **20**, 250 (1955).
3. Kunkel, H. O., Spalding, J. F., de Francis, G., and Futrell, M. F. Cytochrome oxidase activity and body weight in rats and in three species of large animals. *Am. J. Physiol.*, **186**, 203 (1956).
4. Lawrie, R. A. The activity of the cytochrome system in muscle and its relation to myoglobin. *Biochem. J.*, **55**, 298 (1953).
5. Potter, V. R. Tissue homogenates. In Colowick, S. P., and Kaplan, N. O. *Methods in Enzymology*. 1955. Vol. 1, p. 10, Academic Press, New York, N. Y.
6. Schneider, W. C. and Potter, V. R. The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. *J. Biol. Chem.*, **149**, 217 (1943).

PROTECTIVE ROLE OF SHELL MEMBRANES IN BACTERIAL SPOILAGE OF EGGS

J. A. GARIBALDI AND J. L. STOKES
Western Regional Research Laboratory,[a] Albany, California

(Manuscript received December 14, 1957)

The three main barriers which protect the shell egg against bacterial invasion and spoilage are the shell itself, the adjacent shell membranes, and the albumen *(8)*. There is need for additional information on the degree of effectiveness of each of these barriers, on the mechanisms by which they either exclude bacteria or prevent their multiplication, and on the means by which invading bacteria surmount these obstacles.

The present investigations deal with the shell membranes. These parchment-like envelopes which enclose the egg contents are composed of interlacing fibers of a keratin-like protein. The inner membrane is coated on the albumen side with a mucin-like substance which fills the interstices of the fiber network to form an apparently non-porous structure. Despite their thinness, the membranes constitute a tough, flexible envelope. This suggests that the membranes may function as a mechanical barrier to the penetration of bacteria into the egg. There is, however, no direct evidence that the membranes are impermeable to bacteria *(4)*. The suggestion that the membranes have bactericidal properties *(12)* has been shown to be incorrect *(9)*.

Our investigations were undertaken to determine the ability of the egg shell membranes to filter out bacteria, to resist break-through by growing cultures of bacteria, and to support bacterial growth. The extent to which membranes are susceptible to proteolytic digestion by egg spoilage bacteria was also investigated.

MATERIALS AND METHODS

Bacteria. The bacteria used were mainly those isolated by Florian and Trussell *(3)* and classified by them according to their ability to spoil eggs. They included 7 strains of *Pseudomonas fluorescens;* 2 strains of *Pseudomonas putrefaciens;* 2 strains of *Alcaligenes recti;* 6 strains of *Alcaligenes bookeri (a);* 2 strains of *Alcaligenes bookeri (b);* 3 strains of *Alcaligenes faecalis;* 1 strain of *Alcaligenes metalcaligenes;* 3 strains of *Aerobacter cloacae;* 2 strains of *Flavobacterium invisible;* 4 unidentified species of *Flavorbacterium;* 4 strains of *Achromobacter liquifaciens;* 2 unidentified species of *Achromobacter;* 3 strains of *Proteus melanovogenes;* 5 strains of *Proteus vulgaris (a);* 2 strains of *Proteus vulgaris (b);* 2 strains of *Paracolobactrum intermedium (b);* 2 strains of *Paracolobactrum aerogenoides;* 3 strains of *Escherichia freundii;* 1 strain of *Escherichia intermedium;* and 2 unidentified species of the coli-aerogenes group.

The cultures were maintained on trypticase soy agar slants. They were transferred to fresh slants monthly, incubated at 30° C. for 20 to 24 hours and stored at 3° C. until used. Liquid cultures were grown in trypticase soy broth at 30° C. for 20-24 hours and were used as inocula without further treatment. All dilutions used in determining bacterial numbers were made in 0.25% tryptone. Such diluents have been shown to exert a protective action on bacteria *(11)* by preventing a reduction in numbers while the bacterial population is being determined.

Filtration studies. The ability of the egg shell and membranes to remove bacteria from an aqueous suspension was determined with the apparatus shown in Figure 1. The

[a] Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

vacuum filter flask, the test tube, and the crucible adapter were assembled and sterilized at 15 lbs. steam pressure for 15 minutes. The shell alone or the shell with membranes was prepared by immersion of the whole egg in 70% alcohol for several minutes. It was then withdrawn, drained, and flamed to remove the last traces of alcohol. The egg was cracked at the sharp end and the contents were discarded. The inside of the egg shell was rinsed several times with sterile distilled water to remove the albumen adhering to the membranes. To obtain the shell with only the outer membrane present, the inner membrane at the air sac end was removed. To obtain the shell free of all membranes, the inner membrane was removed as described and the outer membrane was carefully peeled and scraped from the shell. The shell with or without the membranes was placed on the crucible adapter. Twenty milliliters of a suspension containing a known number of bacteria per milliliter were added to the inside of the shell. Vacuum equivalent to 46 cm. of mercury was applied to the flask and several milliliters of filtrate were collected. The population in the filtrate was determined by plating appropriate dilutions on trypticase soy agar.

Proteolytic activity. The proteolytic activity of over fifty bacterial strains was determined in the following manner. The test organism was inoculated into sterile 0.2% nutrient broth supplemented with 1% protein, either as casein or ovalbumin. Calcium chloride, 5.5 mg, was added per liter of medium. This is equivalent to 2 mg. of calcium ion per liter. The pH of the medium was adjusted to 9.2 prior to sterilization. After 10 days of incubation at 30°C., the amount of undigested protein was determined by precipitation with trichloracetic acid and measurement of the turbidity produced, in a Klett-Summerson colorimeter with a blue filter. To precipitate the protein from the culture, a series of dilutions was prepared with 1% $(NH_4)_2SO_4$ to contain from 0.04 mg. to 0.4 mg. of protein per milliliter. One tenth of a milliliter of 4 *M* trichloracetic acid was added to 4.9 ml. of each dilution and the turbidity produced was measured within 20 minutes. The concentration of residual, undigested protein in the culture was calculated by comparing its turbidity with that obtained with known concentrations of casein or ovalbumin.

Liberation of ninhydrin positive substances into the culture medium by bacterial action on membrane homogenates was followed by the method of Moore and Stein *(7)*. Aliquots of the culture were prepared for analyses by filtration through an ultrafine sintered glass filter to remove both the membrane particles and the bacteria. Ninhydrin values were obtained by comparison of the color which developed in the test samples with that of a standard leucine solution.

Membrane homogenates. Membranes were peeled from egg shells which had been prepared as previously described. They were disintegrated into very fine particles in a ground-glass homogenizer. The homogenate was sterilized by addition of 1% ethylene oxide at 2° to 4°C. and storage at this temperature for 24 hours. At the end of this period, the homogenate was allowed to warm to room temperature in a hood, in order to remove any residual ethylene oxide. This treatment yielded a sterile membrane preparation. The effect of ethylene oxide, a very reactive reagent, on the membrane material was not investigated. To determine whether the homogenate could serve as a sole source of energy, carbon, and nitrogen for the bacterial strains, it was diluted so as to contain approximately 5 mg. of dry membrane material per milliliter. The homogenate was then supplemented with the following inorganic salts to provide an adequate mineral environment: K_2HPO_4, 10 mg. per liter; $MgSO_4.7H_2O$,200 mg. per liter; $ZnSO_4$,5.0 mg. per liter; $MnCl_2.4H_2O$,7.2 mg. per liter; $FeSO_4.7H_2O$,10.0 mg. per liter; $CaCl_2$,11.0 mg. per liter. The pH was adjusted to 8.7.

Penetration experiments. The apparatus used in these experiments is shown in Figure 2. It consists of a small crystallizing dish, 50 mm. in diameter. It was placed on cotton gauze in the bottom of a petri plate and covered with the top half of a 16-oz. screw top, narrow-mouth bottle. After assembly, the apparatus was sterilized by autoclaving at 15 lbs. pressure for 15 minutes without the egg shell. The egg shell with both inner and outer membranes was prepared as previously described and placed inside the small crystallizing dish. Sterile nutrient broth was added to the inside of the egg shell and to the crystallizing dish. In order to destroy the microorganisms which had escaped the 70% alcohol treatment of the shell and those introduced during the preparation of the shell, the assembled apparatus was treated with ethylene oxide. This was done by placing the entire apparatus in a desiccator which was then evacuated. One milliliter of ethylene oxide was drawn into the evacuated desiccator. After storage for 24 hours at room temperature the desiccator was again evacuated to remove the ethylene oxide and air was allowed to enter through a ster-

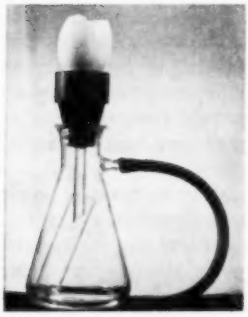


Figure 1. Apparatus for the vacuum filtration of bacteria through shell and membranes.

Figure 2. Apparatus for determining rate of penetration of shell and membranes by bacteria.

ile cotton filter. The apparatus was removed and incubated at 30°C. for several days prior to use to detect any residual contamination. The ethylene oxide treatment proved to be very effective in sterilizing the apparatus. One milliliter of a 20-hour culture of the test organisms was inoculated into the medium in the crystallizing dish and incubated at 30° C. At intervals, samples were taken aseptically from the broth inside the shell to determine when bacterial penetration had occurred.

RESULTS AND DISCUSSION

Filtration through shell and membranes. The ability of the shell and membranes to filter out 3 representative bacteria, *Alcaligenes bookeri (a)*, *Pseudomonas fluorescens* and *Paracolobactrum aerogenoides* from aqueous suspensions is indicated by the data in Table 1. The shell, free of all membrane material, is fully porous and permits unrestricted passage of the bacteria. The filtrates from the shell contained essentially the same number of bacteria as the original suspension. Similar results have been obtained by Haines and Moran *(5)* with *Pseudomonas aeruginosa*. The pores of the shell are too large to hold back bacteria. In freshly laid untreated eggs, the shell may be more retentive. The presence of the proteinaceous bloom or cuticle which blocks the entrances to the pores may help to exclude bacteria temporarily. The shell plus outer membrane, however, is a very good but

TABLE 1

Removal of bacteria from aqueous suspensions by vacuum filtration through egg shell and membranes

	Alcaligenes bookeri (a)	*Pseudomonas fluorescens* No. 7	*Paracolobactrum aerogenoides*
	Bacteria per ml.		
Original suspension	1.87×10^7	2.69×10^7	1.84×10^7
Filtrate through shell only	1.96×10^7	—	2.4×10^7
Filtrate through shell and outer membrane	2.37×10^5	—	4.7×10^5
Filtrate through shell, outer and inner membrane	0	0	0

not a perfect barrier to bacteria. Although 98 to 99% of the bacteria were held back by this combination, hundreds of thousands of bacteria still managed to get through. In contrast, the bacteria were completely stopped when the inner membrane was also present; filtrates from the shell complete with outer and inner membranes were entirely free of bacteria. Indications that bacteria are held back by the shell membrane system were obtained by Walden, Allen, and Trussell *(13)*.

These results indicate that the membranes are very effective mechanical barriers to bacterial penetration. They form a non-porous structure, probably because of the coating of mucin-like material on the inner membrane, which is capable of preventing, at least temporarily, the passage of bacteria.

Proteolysis of membranes and other proteins. Although the membranes are effective bacterial filters, it is obvious from the considerable amount of naturally occurring bacterial egg spoilage, that they do not exclude bacteria, indefinitely, from the egg contents. Also, *Alcaligenes bookeri (a)* and *Pseudomonas fluorescens* (Table 1) have been shown to penetrate the inner membrane of shell eggs and to cause spoilage *(3)*. There must be, therefore, some mechanism by means of which bacteria eventually penetrate the apparently solid membrane barrier. This could be accomplished by growth of the bacteria on the membranes, accompanied by digestion of the membrane material. Suggestive of this mode of attack is our finding that shell membranes kept in contact with suspensions of *Alcaligenes bookeri (a)* for several days, lose their ability to prevent the passage of bacteria. Also, it has been shown that membrane material will support the growth of pseudomonads and other bacteria *(9, 2)* and that colonies will form on membranes of shell eggs infected with *Pseudomonas ovalis (1)*. Since the membranes are composed largely of proteinaceous material, it was thought that the digestion of the membranes might be brought about by proteolytic enzymes secreted by the bacteria. Accordingly, the proteolytic properties of all of the bacteria, egg spoilers and non-egg spoilers, were determined. Data on the digestion of membrane material, ovalbumin, casein and gelatin by representative bacterial cultures are given in Table 2.

The bacterial species varied greatly in protoeolytic properties. *Alcaligenes bookeri (a)* is the most versatile organism. Five of the 6 strains digested, extensively, all of the proteins tested, including the membrane material. In marked contrast, *Escherichia freundii* cannot attack any of the proteins. Between these extremes are the other bacteria which digest some but not all of the proteins.

Surprisingly, *A. bookeri (a)* was the only organism, of all those tested,

TABLE 2

Proteolytic activity of various bacteria isolated from spoiled eggs

Bacterial Strain	Proteolysis [1] of			
	Membrane	Ovalbumin	Casein	Gelatin
Alcaligenes bookeri (a)	+	+	+	+
Proteus melanovogenes	−	+	+	+
Pseudomonas fluorescens (54b)	−	−	+	+
Pseudomonas fluorescens (7)	−	−	−	+
Escherichia freundii	−	−	−	−

[1] + Extensive Digestion; − No Detectable Digestion

which digested membrane material. The progress of the digestion, measured by the appearance of ninhydrin positive substances in the culture filtrates and the increase in bacterial numbers, is shown in Table 3. The ninhydrin positive substances are undoubtedly a mixture of peptides, amino acids and ammonia which arise from the proteolysis of the membrane material. For comparison, the table includes results obtained with *Proteus melanovogenes* which is unable to digest membrane material and is typical of all the other bacteria tested. In this experiment, the membrane particles were suspended in a mineral salt solution and were the sole source of carbon, nitrogen, and energy for the growth of the bacteria.

It is evident from the data in Table 3, that *A. bookeri (a)* increased greatly in numbers with time so that as many as 5 billion cells were present per ml. of membrane homogenate after 96 hours of incubation at 30° C. A population of this density is attained only in highly nutritious culture media. This indicates that the membrane material is an excellent source of nutrients for *A. bookeri*. Concomitantly with the growth of *A. bookeri* there was a marked increase in ninhydrin positive substances in the cultures as the membrane proteins were digested. This increase continued even after the peak of the bacterial population had been passed. At 288 hours, when the experiment was terminated, the amount of protein breakdown products in solution was equivalent to 216 micrograms of leucine per ml. of culture, approximately a 35-fold increase over the amount present initially. There can be no doubt that the growth of *A. bookeri* was at the expense of the membrane proteins.

The results with *P. melanovogenes* were quite different. The bacteria increased to a maximum of 10 million cells per ml in 48 hours, a population much smaller than the billions of cells obtained with *A. bookeri*. Also, there was no significant increase in protein breakdown products in the culture fluid during the experiment. It can be concluded that *P. melanovogenes* is unable to attack the proteins of the shell membranes. The small amount of growth which occurred was probably due to the non-protein constituents of the membranes. Only very small quantities of such substances would be required to support such a small population. It is probably significant for penetration that there are substances in the membranes which support appreciable growth of many bacteria. This may permit the

TABLE 3

Growth on and proteolysis of membrane homogenate

Incubation period (hours)	*A. bookeri (a)*		*P. melanovogenes*	
	Bacteria/ml.	Liberation of ninhydrin positive substances [1]	Bacteria/ml.	Liberation of ninhydrin positive substances [1]
0	1.72×10^4	6.2	1.0×10^4	6.2
24	3.25×10^4	8.2	3.1×10^5	8.4
48	8.4×10^7	21.8	9.7×10^6	5.6
72	7.1×10^8	83.8	9.4×10^6	6.2
96	5.0×10^9	86.3	7.4×10^6	6.2
120	8.6×10^8	129.0	7.1×10^6	6.2
288	6.7×10^8	216.0	—	6.7

[1] Leucine equivalents as micrograms/ml of cell free culture medium.

bacteria to grow directly on the membrane surface, as has been observed by Elliott *(1)*, and to migrate into the egg contents.

The most striking result of these experiments is the great resistance of the membranes to proteolytic attack by bacteria. Only one species, *A. bookeri (a)*, of the many tested was able to digest membrane protein. Many of the other bacterial strains digested ovalbumin, casein, and gelatin but were not able to break down membrane material. The latter consists mainly of a keratin-like protein, a type which is known to be resistant to proteolysis. It appears that extensive proteolytic attack is not the common way by which bacteria penetrate shell membranes. Those occasional bacterial species, which can digest membrane protein may be able to penetrate into eggs more rapidly than non-digesters. The exact mechanism of bacterial penetration of the membranes still remains to be elucidated.

Although there does not appear to be any correlation between bacterial proteolysis and membrane penetration, there is a striking correlation between bacterial proteolysis and egg spoilage. It can be seen in Table 4 that of 23 cultures which Florian and Trussell *(3)* classified as egg spoilers, 19 or 83% are able to attack ovalbumin or casein. In contrast, 25 out of 28 cultures of non-egg spoilers, or 90%, are non-proteolytic. It appears, therefore, that proteolytic ability is important and perhaps decisive in enabling bacteria to establish themselves and multiply in the egg white after penetrating the membranes. The egg white is composed predominantly of native proteins. Their proteolysis would release the nutrients necessary for extensive bacterial growth and, therefore, egg spoilage. Proteolysis by the bacteria could also destroy inhibitors of bacterial growth such as lysozyme, avidin and conalbumin which are proteins present in the egg white. This aspect is under further investigation.

Penetration of membranes. The resistance of shell membranes to penetration by growing cultures of bacteria was determined, as described previously (see Figure 2). Results obtained with 27 cultures which included proteolytic and non-proteolytic bacteria are given in Table 5.

None of the bacterial strains penetrated the membranes within 7 hours. Five of the strains, however, succeeded in penetrating the membranes within 26 hours. Periodically, thereafter, additional strains breached the membranes and at 56 hours, 22 of the 27 strains tested had penetrated. The last 2 strains required 4 days to get through. Thus all of the 27 strains, proteolytic and non-proteolytic, eventually pierced the membranes and reached the broth inside the eggs. In similar experiments Zagaevsky and Lutikova *(14)* obtained penetration of *Bacterium pyocyaneus* and *Bacillus prodigiosus* in 3 days at 37 C.

TABLE 4

Relationship between the ability of bacteria to spoil shell eggs and their ability to produce extracellular proteolytic enzymes

Ability of the bacterial strains to spoil shell eggs		Number of strains found to be	
Classification [1]	Number of strains	Proteolytic [2]	Non-proteolytic
Positive	23	19	4
Negative	28	3	25
Variable	5	2	3

[1] According to Florian and Trussell (3).
[2] Based on the ability to digest casein and/or ovalbumin.

TABLE 5

Rate of penetration of bacteria through the shell and membranes

Hours of incubation	Number of bacterial strains [1] which had penetrated
7	0
26	5
48	17
56	22
72	24
79	25
97	27

[1] Of a total of 27 tested; includes species of *Aerobacter, Proteus, Alcaligenes, Pseudomonas, Flavobacterium, Escherichia* and *Achromobacter.*

whereas *Sarcina flava* did not penetrate in 5 days. Elliot *(1)* reported that when *Pseudomonas ovalis* was placed in contact with the inner membrane of normal shell eggs, the time for penetration varied from one to more than 8 days.

These results support the conclusion from the proteolysis experiments that extensive proteolytic attack by the bacteria is not necessary for penetration, since non-proteolytic strains also penetrate the membranes. Moreover, there was no apparent correlation between the rate of penetration for a particular strain and its proteolytic properties.

It should be emphasized that in the above type of experiment, the membranes are under severe microbial attack. The outside of the shell-membrane system is in contact with billions of bacteria and the membrane surfaces are bathed in nutrient fluid. Even if only a single bacterial cell penetrated, it would multiply rapidly in the inner nutrient broth and give a positive result. Under more natural conditions, penetration of the shell membranes is undoubtedly a much slower process *(14, 4, 6, 10)*. Also, in the whole egg, the single bacterial cell or small group of cells which penetrate the membranes may die out in the unfavorable environment of the albumen. Thus the membranes may also protect the egg against bacterial spoilage by lessening the burden placed on the defenses of the albumen.

SUMMARY

The egg shell, free of membrane material, cannot restrain the passage of bacteria when the latter are filtered through the shell. In contrast, the outer shell membrane removes 98 to 99% of the bacteria and the combination of outer and inner membranes removes all of the bacteria from aqueous suspensions which contain as many as 20 million bacteria per ml. The membranes, therefore, are efficient mechanical barriers to the passage of bacteria under the experimental conditions used.

The membranes are very resistant to proteolytic attack by bacteria. Only one species, *Alcaligenes bookeri (a)*, grew extensively in membrane homogenates and digested the membrane proteins. Many of the other bacteria tested, which included species and strains of *Pseudomonas, Proteus, Alcaligenes, Aerobacter,* and *Escherichia,* digested proteins such as ovalbumin, casein and gelatin but not the highly resistant keratin-like protein of the membranes. Moreover, all of the bacterial species, proteolytic and nonproteolytic, penetrated the shell membranes within 1 to 4 days when the latter, supported by

the shell, were immersed in growing cultures of the bacteria. These results suggest that the bacterial penetration of shell membranes does not involve extensive proteolytic attack.

A close correlation exists between the ability of bacteria to digest proteins and to spoil shell eggs. Proteolysis may be important in enabling bacteria to establish themselves and multiply in the albumen.

Acknowledgment

The authors wish to thank Dr. P. C. Trussell for cultures used in this study.

LITERATURE CITED

1. Elliott, R. P. Spoilage of shell eggs by *Pseudomonads*. *Appl. Microbiol.*, **2**, 158 (1954).
2. Elliott, L. E., and Brant, A. W. Effect of saline and egg shell membrane on bacterial growth. *Food Research*, **22**, 241 (1957).
3. Florian, M. L. E., and Trussell, P. C. Bacterial spoilage of shell eggs. IV. Identification of spoilage organisms. *Food Technol.*, **11**, 56 (1957).
4. Gillespie, J. M., and Scott, W. J. Studies in the preservation of shell eggs. IV. Experiments in the mode of infection by bacteria. *Aust J. Appl. Sci.*, **1**, 514 (1950).
5. Haines, R. B., and Moran, T. Porosity of, and bacterial invasion through, the shell of the hen's egg. *J. Hyg.* **40**, 453 (1940).
6. Miller, W. A., and Crawford, L. B. Some factors influencing bacterial penetration of eggs. *Poultry Sci.*, **32**, 303 (1953).
7. Moore, S., and Stein, W. H. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.*, **176**, 367 (1948).
8. Romanoff, A. L., and Romanoff, A. J. The Avian Egg. (1949). John Wiley and Sons, Inc., New York, N. Y.
9. Stokes, J. L., and Osborne, W. W. Effect of the egg shell membrane on bacteria. *Food Research*, **21**, 264 (1956).
10. Stokes, J. L., Osborne, W. W., and Bayne, H. G. Penetration and growth of *Salmonella* in shell eggs. *Food Research*, **21**, 510 (1956).
11. Straka, R. P., and Stokes, J. L. Rapid destruction of bacteria in commonly used diluents and its elimination. *Appl. Microbiol.*, **5**, 21 (1957).
12. Stuart, L. S., and McNally, E. H. Bacteriological studies on the egg shell. *U. S. Egg and Poultry Mag.*, **49**, 28 (1943).
13. Walden, C. C., Allen, I. V. F., and Trussell, P. C. The role of the egg shell and shell membranes in restraining the entry of microorganisms. *Poultry Sci.*, **35**, 1190 (1956).
14. Zagaevsky, J. S., and Lutikova, P. O. Bacteriological study of the egg. *U. S. Egg and Poultry Mag.*, **50**, 121 (1944).

MICROBIAL FLORA OF PACKAGED FRANKFURTERS AND THEIR RADIATION RESISTANCE[a]

SUZANNE DEWEES DRAKE, JAMES B. EVANS, AND C. F. NIVEN, JR.
Division of Bacteriology, American Meat Institute Foundation, and Department of Microbiology, The University of Chicago, Chicago, Illinois

(Manuscript received December 7, 1957)

It has been reported previously from this laboratory *(13)* that the bacteria primarily responsible for the spoilage of refrigerated fresh beef are pseudomonads that are relatively sensitive to high energy radiations. When these organisms are killed by low doses of irradiation the refrigerated shelf-life of the meat is extended several-fold, but ultimately it spoils due to microorganisms that grow much more slowly at low temperatures than do the pseudomonads. Prominent among the more radiation-resistant spoilage microorganisms was a gram-positive organism which appeared to be identical to *Microbacterium thermosphactum*, as described by McLean and Sulzbacher *(7)*.

The present report concerns a similar study of the microbial flora of packaged frankfurters before and after irradiation. As a result of the bacteriostatic effect of curing ingredients, the bacteria present on frankfurters are predominantly gram-positive types. Ogilvy and Ayres *(9)* have reported that micrococci, bacilli, and sarcinae were most numerous, while lactobacilli and gram-negative bacteria were present in smaller numbers. Also, appreciable numbers of yeasts sometimes were encountered. They also studied the effect of increased CO_2 tension on the flora and found that as the CO_2 concentration was increased molds, yeasts, and micrococci were progressively inhibited and lactobacilli became predominant.

EXPERIMENTAL METHODS

Cellophane-wrapped frankfurters were purchased in local retail stores. Thirty-six packages from 11 different companies were examined. Duplicate samples were incubated at 2° and 10° C. until surface sliming became evident. Some of this slime was scraped off with a sterile scalpel, diluted in distilled water, and plated in APT agar *(4)*. The plates were incubated for 5 days at 25° C. Ten representative colonies were picked from the appropriate dilution of each sample, and bacteria were identified according to methods in the *Manual of Methods for Pure Culture Study of Bacteria (2)*. Yeasts were cultivated in MY broth *(5)* and identified according to the methods of Wickerham *(10–12)* and Lodder and Van Rij *(6)*.

The radiation resistance of pure cultures was determined as previously described *(13)*. A number of experiments were conducted to determine the effect of irradiation on the naturally-occurring microbial flora of frankfurters. Frankfurters from local retail stores were cut longitudinally and vertically to make 4 samples from the same frankfurter. Two of the quarters were individually wrapped in oxygen-permeable, moisture-proof cellophane and the other two were vacuum packaged in impermeable cellophane-polyethylene bags. After irradiation the samples were placed in a covered pyrex dish containing a beaker of water to supply a high humidity. These dishes were then held at 10° C. or at

[a] Journal Paper No. 151, American Meat Institute Foundation. This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 807 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

2° C. The bacterial counts were determined immediately after irradiation and after varying intervals of incubation. Colonies were picked and identified as previously described.

RESULTS AND DISCUSSION

Microbial flora of the slime on refrigerated frankfurters. A total of 353 microorganisms were isolated from 36 packages of frankfurters and were tentatively identified. Of these, 275 were bacteria and 78 were yeasts. The results are summarized in Table 1. Microbacteria and yeasts constituted the

TABLE 1

Microorganisms in the surface slime of refrigerated frankfurters

Isolates	From frankfurters stored at		Total number
	2° C.	10° C.	
Lactobacilli (heterofermentative)	14	28	42
Lactobacilli (homofermentative)	11	13	24
Leuconostocs	12	32	44
Microbacteria	61	41	102
Micrococci	11	29	40
Streptococci	4	8	12
Yeasts	48	30	78
Gram-negative rods	6	5	11
Total	167	186	353

two most frequently isolated groups of organisms and both of these were more common on franks stored at 2° C. than on those stored at 10° C. At 10° C., lactobacilli, leuconostocs, and micrococci were of greater relative significance.

Eighty-three representative bacteria were studied more extensively. Twenty-three strains of microbacteria were studied, and all proved to be very similar or identical to *Microbacterium thermosphactum (7, 13)*. The only differences noted were the production of acid from glycerol by 15 strains, the fermentation of lactose by 3 strains, and the fermentation of galactose by 6 strains.

Twenty-four strains of lactobacilli were studied (16 heterofermentative strains and 8 homofermentative strains). All of the lactobacilli grew at 2° C., and 12 of the heterofermentative and 6 of the homofermentative strains also grew at 45° C. All of the lactobacilli could produce hydrogen peroxide during growth, and all could cause greening of Vienna sausage. Most of the heterofermentative strains were identical with *Lactobacillus viridescens (8)*. The homofermentative strains did not coincide well with any generally recognized species *(1)*.

Fifteen *Leuconostoc* strains were studied. Eleven of them produced a polysaccharide slime from sucrose and 12 of them produced hydrogen peroxide. They all grew at 2° C. and 30° C., but only one grew at 45° C. Two strains were identified as *Leuconostoc mesenteroides* and the remaining 13 as *Leuconostoc dextranicum (1)*.

The 6 streptococci appeared to be enterococci, but differed from each other and from described species in a number of physiological tests. The 12 micrococci were extremely heterogeneous, but one proved to be a typical coagulase-positive *Staphylococcus aureus*. Three tetracocci were studied and were quite similar, but they were not identified specifically.

The gram-negative rods were not studied since they appeared to be of little significance, but they appeared to be pseudomonads.

The yeasts were studied quite extensively, and will be the subject of a separate report.

Radiation resistance of the microbial flora of frankfurters. The radiation resistance of 40 representative microorganisms is presented in Table 2. All of these were considerably more resistant than the pseudomonads from fresh meats, which had an LD_{99} of about 6500 rads under similar conditions. Most of these organisms from frankfurters were of the type that grow in chains or clumps and showed the multi-hit type of survival curve. The radiation resistance of some of these organisms was determined several different times, and the results agreed within approximately 11%.

TABLE 2

Radiation resistance of representative microorganisms from the surface of frankfurters

Organism	No. of strains	LD_{99}[1] rads x 10^{-3}
Lactobacilli (heterofermentative)	4	43–74
Lactobacilli (homofermentative)	3	172–209
Leuconostocs	5	40–113
Microbacteria	4	61–89
Micrococci	3	67–98
Staphylococcus aureus	1	44
Streptococci	3	81–236
Tetracocci	2	173–215
Yeasts	15	49–257

[1] Dosage for 99% destruction in TY broth (1.0% tryptone and 0.5% yeast extract).

Irradiation of frankfurters. The franks purchased in local grocery stores generally had an initial population of 10^4 to 10^6 microorganisms per gram. However, some packages exhibited very low counts. These franks were packaged in cellophane or were vacuum packaged, and irradiated as described under Experimental Methods. Seven experiments of this type were conducted, and the results with the cellophane-wrapped franks in four of these are summarized in Table 3. The results of the other three experiments were very similar, and the results with vacuum-packaged franks were not significantly different from those that were cellophane wrapped. However, the vacuum-packaged samples had a much better texture and color after prolonged storage even though conditions were quite humid.

It can be noted that an irradiation dosage of 140,000 rads reduced the initial microbial population about 99%. This appeared to delay microbial spoilage for 2 or 3 days at 10° C., and somewhat longer at 2° C. However, even this level of irradiation adversely affected the color of the product in both types of packages, especially the cut surface. These results are consistent with the results of Erdman and Watts *(3)* who reported that 186,000 rads adversely affected the color of bologna and was inadequate to extend the keeping time at room temperature.

Experiment No. 6 (Table 3) illustrates the importance of the initial population level. These franks had a very low microbial population before irradiation, and 230,000 rads killed virtually all of the organisms that could grow at 2° C. They showed no growth after 3 weeks, but after 5 weeks' incubation

TABLE 3

Effect of irradiation and storage on the microbial population of cellophane-packed frankfurters

Experiment No. and storage temp.	Irradiation (rads)	Bacterial count after			
		0 days	5 days	10 days	21 days
No. 2	0	7200	33×10^7	36×10^7	
	140,000	600	50×10^6	59×10^7	
10° C.	230,000	140	10×10^6	30×10^7	
	460,000	30	11×10^4	10×10^6	
No. 3	0	82×10^4	18×10^7	99×10^7	
	140,000	78×10^2	18×10^6	31×10^7	
10° C.	230,000	21×10^2	47×10^5	33×10^7	
No. 6	0	200	3300	11×10^5	27×10^7
2° C.	140,000	<10	<10	6200	24×10^7
	230,000	<10	<10	<10	<10
No. 7	0	94×10^5	37×10^7	11×10^8	18×10^8
	140,000	29×10^4	19×10^5	72×10^5	26×10^7
2° C.	230,000	11×10^4	66×10^4	13×10^6	19×10^7

these samples had reached 10^6 organisms per gram. The franks in experiment No. 2 had a fairly low initial population, but 460,000 rads only approximately doubled their shelf-life at 10° C.

Microbial flora of irradiated frankfurters. Five representative colonies were picked from the plates for each sample in the experiments discussed in the preceding section. These organisms were then generally identified as to genus. Typical results from the same four experiments listed in Table 3 are summarized in Table 4. The flora of the unirradiated samples were similar to those shown in Table 1. The irradiated franks showed a predominance of yeast and sporeforming bacilli. Other levels of irradiation and the other experiments also gave similar results. Thus, the selective action of these moderate levels of irradiation on the microbial flora coincides with the information presented in Tables 1 and 2 concerning the initial flora and their relative radiation resistance.

SUMMARY

The microbial flora of the surface slime of refrigerated frankfurters has been shown to consist primarily of lactic acid bacteria, yeasts, and micrococci. The most common bacteria were members of the species *Microbacterium thermosphactum*, followed by lactobacilli, leuconostocs, micrococci, and streptococci. Small numbers of gram-negative rods occasionally were found. The radiation resistance of these organisms was determined, and was found to range from about 40,000 rads to 250,000 rads to destroy 99% of the organisms in a broth suspension. The most resistant strains included some yeasts, some streptococci, tetracocci, and homofermentative lactobacilli.

Irradiation of commercially produced frankfurters indicated that 140,000 rads reduced the initial microbial population about 99%, but even this dosage adversely affected the appearance of the product, and did not markedly delay microbial spoilage at 10° C. Irradiation with 460,000 rads approximately

TABLE 4

Microbial flora of irradiated frankfurters

Experiment No. and storage temp.	Irradiation (rads)	Microbial flora[1]	
		Cellophane packed	Vacuum packed
No. 2 10° C.	0	2 Lactobacilli 1 Bacillus 1 Yeast 1 Microbacterium	2 Leuconostocs 2 Micrococci 1 Microbacterium
	230,000	4 Bacilli 1 Yeast	4 Bacilli 1 Yeast
	460,000	5 Bacilli	2 Bacilli 2 Yeasts 1 Tetracoccus
No. 3 10° C.	0	2 Lactobacilli 1 Micrococcus 1 Streptococcus 1 Yeast	3 Yeasts 1 Leuconostoc 1 Tetracoccus
	230,000	5 Yeasts	3 Yeasts 2 Leuconostocs
No. 6 2° C.	0	3 Yeasts 2 Leuconostocs	4 Leuconostocs 1 Yeast
	230,000	5 Yeasts	5 Yeasts
No. 7 2° C.	0	5 Microbacteria	2 Microbacteria 2 Leuconostocs 1 Bacillus
	230,000	5 Bacilli	3 Streptococci 2 Microbacteria

[1] Identity of five isolates.

doubled the time before microbial spoilage at 10° C. However, if the initial microbial population was very low, a dosage of 230,000 rads could greatly delay microbial spoilage at 2° C. The flora developing on the irradiated frankfurters were predominantly yeasts and members of the genus *Bacillus*.

LITERATURE CITED

1. Breed, R. S., Murray, E. G. D., and Hitchens, A. P. *Bergey's Manual of Determinative Bacteriology*. 6th ed., 1948, Williams and Wilkins, Baltimore, Md.
2. Committee on Bacteriological Technic, Society of American Bacteriologists. *Manual of Methods for Pure Culture Study of Bacteria*. Biotech Publications, Geneva, N. Y.
3. Erdman, A. M., and Watts, B. M. Radiation preservation of cured meats. *Food Technol.*, **11**, 349 (1957).
4. Evans, J. B., and Niven, C. F., Jr. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. *J. Bacteriol.*, **62**, 599 (1951).
5. Haynes, W. C., Wickerham, L. J., and Hesseltine, C. W. Maintenance of cultures of industrially important microorganisms. *Applied Microbiol.*, **3**, 361 (1955).
6. Lodder, J., and Kreger-van Rij, N. J. W. *The Yeasts, A Taxonomic Study*. North Holland Publishing Co., Amsterdam, Holland.
7. McLean, R. A., and Sulzbacher, W. L. *Microbacterium thermosphactum*, spec. nov.: a nonheat resistant bacterium from fresh pork sausage. *J. Bacteriol.*, **65**, 428 (1953).

8. NIVEN, C. F., JR., AND EVANS, J. B. *Lactobacillus viridescens*, nov. spec., a heterofermentative species that produces a green discoloration of cured meat pigments. *J. Bacteriol.*, **73**, 758 (1957).

9. OGILVY, W. S., AND AYRES, J. C. Post-mortem changes in stored meats. V. Effect of carbon dioxide on microbial growth on stored frankfurters and characteristics of some microorganisms isolated from them. *Food Research*, **18**, 121 (1953).

10. WICKERHAM, L. J. A critical evaluation of nitrogen assimilation tests used in the classification of yeasts. *J. Bacteriol.*, **52**, 293 (1946).

11. WICKERHAM, L. J. *Taxonomy of Yeasts.* Tech. Bulletin No. 1029, U. S. Dept. of Agriculture, Washington, D. C. (1954).

12. WICKERHAM, L. J., AND BURTON, K. A. Carbon assimilation tests for the classification of yeasts. *J. Bacteriol.*, **56**, 363 (1948).

13. WOLIN, E. F., EVANS, J. B., AND NIVEN, C. F., JR. The microbiology of fresh and irradiated beef. *Food Research*, **22**, 682 (1957).

PRETREATMENT OF BRANDY STILLAGE FOR DISPOSAL IN A MUNICIPAL WASTE TREATMENT PLANT

REESE H. VAUGHN, GEORGE K. YORK II, MELVIN F. KLEMMICK, AND L. DOUGLAS DAVIS

Department of Food Technology, University of California, Davis, California
Sonoma Valley County Sanitation District, Sonoma, California
and
Samuele Sebastiani Winery, Sonoma, California

(Manuscript received October 31, 1957)

A winery was faced with the problem of either ceasing brandy distillation or providing for adequate pretreatment of the approximately 50,000 gallons per day (g.p.d.) of stillage being sent to a newly constructed disposal system. This disposal plant, designed exclusively for treating domestic wastes, is a conventional biological filter system with one recirculating filter, one anaerobic digestion tank, one open digestion tank (still uncovered because of lack of funds), and primary and secondary clarifiers. The final effluent is chlorinated. The plant has a designed hydraulic capacity of 3 m.g.p.d. It registered an average flow of 0.75 m.g.p.d. during the summer and fall of 1956 (dry season) and 1.5 m.g.p.d. during the winter and spring of 1956–1957 (wet season).

The winery is located in an urban area, making conditions unsuitable for intermittent irrigation disposal of the waste *(2)*. The urgency of the problem required the translation of knowledge gained over the years in laboratory and small pilot scale experiments to full scale treatment in the existing system.

The first attempt at pretreatment of the stillage at the winery was simple liming in order to control the pH of the waste to near neutrality. Although this control afforded some initial relief during the 1954–1955 season when the disposal system was being broken in, it was also certain that additional treatment of the stillage would be required. It was speculated that this disposal system would handle the stillage if it was properly detartrated, diluted with condenser water from the still, and then further diluted with domestic sewage during passage through approximately 3 miles of sewerage system before arrival at the headworks. This speculation was based on the knowledge that proper detartration would reduce the potential pollution load, as measured by biological oxygen demand (B.O.D., 5 days at 20° C.), by 50% to 75% and greatly reduce, if not eliminate, the buffering capacity of the stillage. Furthermore, simple dilution of the treated stillage would reduce the load on the system. It was felt that the increased volume resulting from dilution would not seriously overload the capacity of the disposal unit under normal conditions of operation.

Some of the results of these more detailed investigations will be described.

METHODS

Sampling. Stillage samples, both treated and untreated, were collected after distillation and dilution with a constant volume of condenser water. Sewage samples, including stillage or not, were taken at 2-hour intervals from 4 points in the plant: (a) raw sewage from the headworks, (b) effluent from the primary clarifier, (c) effluent from the trickling

filter, and (d) unchlorinated final effluent from the secondary clarifier. A complete series of samples were collected once a week. The day of collection was changed from week to week. Although 24-hour composites were taken on occasion, the collection period generally extended over 16 hours (8 A.M. to 12 midnight). The plant operates unattended throughout the "graveyard" shift. The volume of sewage influent and its rate of flow through the plant were recorded as each sample was taken. The pH value was run on a portion of each sample. Then, if not used immediately, samples were held at 4° C. until analyzed. Composite 16-hour samples were made, using corrections for variations in volume that occurred at different times during the day of collection.

Control analyses. The biochemical oxygen demand (B.O.D.) was selected as the index of organic loading of the disposal system. These determinations were made according to the *Standard Methods* of the American Public Health Association for the sodium azide modification *(1)*. Incubation was for 5 days at 20° ± 1° C.

Total, settleable, and suspended solids, 3 other useful indicators of the efficiency of purification of the waste, also were determined by the procedures recommended in *Standard Methods (1)*.

Tartrate analyses by the sodium metavanadate method *(6)* were used to determine the amount of tartrate removed from the stillage and the purity of the calcium tartrate recovered.

The apparent efficiency of each step in treatment of the waste was determined by calculation of the % reduction in B.O.D. or solids (including tartrate) as compared with the untreated material. To follow the waste through the plant, calculations were made on the basis of a 2-hour retention time at each station. The retention time naturally varied in proportion to the rate of flow of the influent sewage but was found to average 2 hours during a 24-hour period of operation. Facilities for recirculation and an automatic by-pass for volumes over the hydraulic capacity of the plant permitted some control over the retention time.

Pretreatment of stillage to remove tartrate. The pH of California brandy stillage generally is 3.5 or above so that the tartrate is present almost entirely as potassium bitartrate (cream of tartar) *(4)*. This soluble salt is precipitated as calcium tartrate when calcium hydroxide, added as lime in the presence of soluble calcium ion as calcium chloride, is used to neutralize the stillage. Otherwise, if calcium chloride is omitted, only about half of the bitartrate will be removed as calcium tartrate. The other half will remain in solution as the very soluble potassium tartrate. The necessity for addition of the soluble calcium salt is illustrated by the two equations:

$$2K\,HC_4H_4O_6 + Ca(OH)_2 \longrightarrow CaC_4H_4O_6 + K_2C_4H_4O_6 + 2H_2O \qquad (1)$$

$$K_2C_4H_4O_6 + CaCl_2 \longrightarrow CaC_4H_4O_6 + 2KCl \qquad (2)$$

The methods described by Marsh *(3, 4)* were used to determine the amounts of lime and calcium chloride required to precipitate the calcium tartrate at a controlled pH value of 4.5 ± on a batch-wise basis.

After the supernatant stillage is decanted and discharged into the sewer, the calcium tartrate precipitate is washed and dried *(4)*. Although some loss of tartrate results from this method of precipitation, experience has shown that neutralization of the stillage to approximately pH 4.5 results in a salt of higher purity. Even though more tartrate is precipitated at higher pH values, the isoelectric points of many of the extraneous materials held in suspension are reached, thus causing their contaminating precipitation. At first sight nearly complete neutralization would appear to be advantageous, both from the standpoint of tartrate recovery and stillage disposal as related to total solids removal. However, such is not the case when the stillage has to be pretreated at the winery and calcium tartrate must be recovered in order to help pay the costs of pretreatment. The high pH neutralization procedure actually is detrimental. Non-tartrate materials are very difficult to remove from the calcium tartrate by washing, and a lower purity tartrate results. In addition the increased washing required causes an increase in the volume of polluted wash water to be treated at the disposal plant.

Two types of materials are distilled at the winery: (a) conventional wine "distilling material" or "D.M."; and (b) lees, the semiliquid residues resulting from natural sedimentation of solids from table and dessert wines, or from refrigeration or clarification and

filtration sediments. Lees containing more than 6 to 8% alcohol are diluted to this level with water or low alcohol DM before distillation in order to insure maximum performance of the still.

Tartrate removal from these different distilling materials was tested in 3 ways: (a) precipitation of the tartrates in the material before distillation—"cold throw"; (b) precipitation of the tartrates in hot stillage after racking (sedimentation)—"hot throw," and (c) removal of the tartrates before distillation as potassium bitartrate by differential centrifugation of the lees with a Sharples continuous centrifuge, model Super-D-canter, PY-14-cylindrical, operated at a rate of 1,100 to 1,500 g.p.h. The centrifuge detartrated stillage was neutralized to at least pH 6.0 before it was discharged into the sewer.

Calcium tartrate was removed on a batch-wise basis. Each treatment involved approximately 14,000 gallons of liquid. With the "hot throw" precipitation the hot stillage normally was allowed to stand for 8 to 10 hours to permit sedimentation of settleable solids prior to recovery of tartrate from the decanted, cleared supernatant. Sometimes, for convenience, the settling period was extended to 24 hours before racking. The sediment remaining after racking the hot stillage was brought to pH 4.5 to 4.7 with lime and metered into the sewer line over a 10-hour period. This pumpable sediment constituted 10 to 15% of the total volume of each batch of stillage collected for treatment by the "hot throw" method.

RESULTS

Although apparently not anticipated as a major problem, it suddenly became clear during the 1954-1955 distillation season, when the treatment plant first was being put into operation, that the 50,000 gallons of untreated stillage received in addition to the normal load of domestic waste during 10 hours per day seriously hindered efficient operation. Impairment was particularly noticeable in connection with starting the digester which had been designed to accumulate enough gas to provide the sludge heater with fuel on a continuous operating basis. However, before enough digester gas was produced daily to maintain the temperature of the heater without the use of "bottle" gas, 110 days had elapsed. Undesirable odor development also indicated serious malfunctioning of the plant. Data in Table 1 illustrate the degree of inefficient functioning of the plant. The effect of untreated stillage on digester gas production is particularly striking. The low pH values and the poor "over-all" reduction in potential pollution of the mixed domestic waste and untreated stillage as measured by 5-day B.O.D. determinations also reflect the inefficiency of the treatment during this critical "shakedown" period.

As an emergency measure, until equipment for tartrate removal could be assembled at the winery, the stillage was neutralized with lime before discharge into the sewerage system. Although this simple liming helped to control the pH of the mixed waste, the mandatory improvement in the "over-all" efficiency of the plant was not forthcoming. It was necessary to add lime to the digester. A total of 3000 pounds was added between December, 1954, and April, 1955. An additional 200 pounds of ammonium sulfate were added as an accessory food to favor desirable microbial activity during this period. Finally, in April, 1955, the digester reached an acceptable activity and the treatment plant, handling only domestic waste, came into balanced operation (Table 1), probably because distillation of the wines prepared during the harvest season had been completed. Henceforth, only stillage which had been treated for tartrate removal was discharged into the system.

Reduction in B.O.D. as influenced by method of detartration. It had been established in previous studies *(5, 8)* that detartration of stillage would

TABLE 1

Effect of treated and untreated stillage mixed with domestic waste on B.O.D. reduction, pH value, and volume of digester gas produced at the Sanitation District treatment plant

Sample station	Raw influent		Primary clarifier effluent		Filter effluent		Final clarifier effluent		pH of sludge	Gas production cu. ft. per day
Type of waste	pH	B.O.D.,[1] p.p.m.	B.O.D.,[1] p.p.m.	% [2] removed	B.O.D.,[1] p.p.m.	% [2] removed	B.O.D.,[1] p.p.m.	% [2] removed		
Domestic sewage	7.0	292	207	29	104	64	27	91	6.2	16,000
Domestic sewage and untreated stillage	5.1	2100	1850	13	1200	43	960	54	4.1	2,100
Domestic sewage and treated stillage [3]	6.9	714	502	30	201	72	86	88	6.1	20,000

[1] Average 5-day B.O.D. at 20° C.
[2] Over-all reduction at each sample station as compared with raw influent.
[3] Three year average for combined detartration methods "cold throw," "hot throw," and centrifugation, respectively.

TABLE 2

Effect of detartration on reduction in B.O.D. values of undiluted brandy stillage

Type of stillage	pH	5-day B.O.D., p.p.m., 20° C.		
		Maximum	Minimum	Average
Untreated D.M. stillage	4.0	3,600	1,700	2,200
Detartrated D.M. stillage	4.6	1,700	800	1,100
B.O.D. removed, %	....	52.8	53.0	50.0
Untreated lees stillage	3.9	38,000	2,000	18,000
Detartrated lees stillage	4.5	11,000	1,200	3,400
B.O.D. removed, %	....	71.0	40.0	81.1

remove between 50% and 75%of the potential pollution load of the waste as measured by the 5-day B.O.D. Results shown in Table 2 confirm the earlier investigations on the effects of detartration insofar as over-all reduction in B.O.D. is concerned. However, in the present study where more samples were tested over a longer period of time, it appears that detartration of ordinary fermentation (wine) stillage can be expected to remove about 50% of the 5-day B.O.D. whereas with lees stillage the removal ranges from 50% up to more than 75% of the original B.O.D. The difference in % reduction in B.O.D. must result from the difference in solids content of the two types of stillage. On purely theoretical grounds, as already discussed by Vaughn and Marsh *(10)*, the difference in tartrate content alone could account for the higher % reduction in B.O.D. for most of the lees samples. Conventional stillage contains from as little as 0.10% up to about 0.75% bitartrate, whereas racking lees stillage contains from 2 to 3% and combined clarification and refrigeration lees stillage contains from 25 to 30% cream of tartar. However, this probably is not the case because lees stillage always contains more suspended solids than does ordinary D.M. stillage.

Even with the marked reduction in potential pollution and oxygen demand effected by detartration it was certain that the detartrated stillage, regardless of origin, still was too concentrated to be handled by the disposal system. Therefore, simple dilution was resorted to in order to reduce the B.O.D. values to a level that could be treated effectively in the plant. For this purpose it was proposed to use the constant volume of condenser water used for distillation plus all waste water available at the winery and count on further dilution with domestic sewage while the detartrated, diluted stillage traveled through approximately 3 miles of sewerage system from the winery to the headworks of the treatment plant. The dilution of the detartrated stillage at the winery was approximately 3 parts of water to 1 part of stillage, a dilution which obtained with slight fluctuations throughout the distillation season (from September through June, on an intermittent basis after the grape harvest). The additional dilution effected in the sewerage system is unpredictable even though the average daily flow is measured at the plant because part of the dilution is caused by infiltration of storm waters into the system during the rainy season, the time of the year much of the distillation is done. In any event, detartration by either of the 3 methods plus dilution at the winery provided stillage wastes that were more amenable to stabilization when mixed with domestic sewage in the disposal system. However, as shown in Table 3, the "hot throw" method of detartration apparently resulted in a waste

TABLE 3

Effect of method of detartration on reduction in B.O.D. values and suspended solids in treated stillage-sewage mixtures

Method of detartration	5-day B.O.D., p.p.m.[1]		% removed	Suspended solids, p.p.m.		% removed
	Raw influent	Final effluent		Raw influent	Final effluent	
"Cold throw"	970	340	65	520	119	77
"Hot throw"	420	43	90	438	88	80
Centrifugation	512	93	82	714	186	74

[1] Incubated at 20° C.

that was easier to purify. The "cold throw" method of detartration gave good results with straight D. M. but not with lees, because of the increased solids content of the latter. Continuous centrifugation removed the bitartrate well but increased the solids content in the raw influent to an objectionable level. As can be seen in the table, the increase in solids is also reflected in the lower over-all reduction in the 5-day B.O.D. value of the centrifuge stillage—domestic sewage mixture after treatment in the disposal plant. Just why centrifugation should cause the observed increase in solids is conjectural.

DISCUSSION

Although projection of the results of small scale experiments to full scale treatment of the stillage without recourse to intermediate pilot studies did relieve the emergency a number of problems remain to be investigated. The addition of 150,000 gallons of water as a diluent for the detartrated stillage is a wasteful load on the hydraulic capacity of the treatment plant. This situation might be relieved for the present simply by metering the diluted stillage into the sewerage system over a 24-hour period instead of discharging it all in 10 hours as is done now. The rate of flow of sewage during the night is low. However, the influent waste at night also is correspondingly less polluted so more favorable dilution of the stillage should also result in a favorable increase in the biological efficiency of the plant.

In the event that a large increase in population occurs it will be mandatory to find another way to pretreat the stillage. Several possibilities exist. A predigestion system could be installed at the winery. The possible use of such pretreatment was pointed out by Vaughn and Marsh *(9)* who found that simple liming of the stillage to pH 7 followed by mesophilic, anaerobic digestion for 24 hours at 35° C. (95° F.) caused a reduction in 5-day B.O.D. values of the waste (settled effluents) that ranged between 70% and 75%. Pearson, Feuerstein, and Onodera *(7)* also obtained similar results. These latter authors also suggested that the gases obtained from stillage digestions be trapped and burned in the boiler supplying steam for the still. The known vagaries of anaerobic bacterial digestions coupled with the known intermittent production of stillage with quite variable composition combine to emphasize the necessity for caution in deciding if gas is to be collected for use at the winery. The present authors believe this operation can be done better at the disposal plant.

More efficient removal of solids from stillage prior to its treatment at the disposal plant also must be considered. The notoriously light and bulky solids that result from detartration, lime flocculation, etc., still remain a problem

because of their capacity to resist conventional compaction systems. Alum flocculation gives acceptable results but the quantities of chemicals required make this method of pretreatment uneconomical. If centrifugal removal of potassium bitartrate becomes the accepted method for detartration then vacuum filtration of lime flocculated (pH *7 vs.* pH 11) as well as chemically detartrated stillage must be investigated.

Too little is known of the effects of direct aeration on purification of stillage to permit speculation. Therefore, this method of solids destruction and removal must also be studied in detail.

SUMMARY

Circumstances arose that required treatment of brandy stillage in a disposal plant designed exclusively for conventional treatment of municipal wastes.

Successful treatment of the stillage in the disposal plant was accomplished after prior detartration of the distilling material or still effluent and increase of its pH at the winery. Then the detartrated waste was diluted with still condenser water and waste wash water (3 parts water to 1 part stillage) before leaving the winery. The diluted stillage received further dilution with domestic waste in its travel through about 3 miles of sewerage system between the winery and the treatment plant.

The method of detartration influenced the over-all efficiency of the purification system. The over-all reduction in 5-day B.O.D. of the stillage-domestic sewage mixture accomplished by the treatment plant was 65% for the "cold throw" method as compared with 90% for the "hot throw" method of chemical detartration with $Ca(OH)_2$ and $CaCl_2$ and 82% for the centrifugation method. During the three-year period of observation of both conventional stillage and lees stillage, the average over-all reduction in 5-day B.O.D. of the stillage-domestic sewage mixture, including all methods of detartration, accomplished by the treatment plant was 88%. When the system handled domestic waste alone, the over-all reduction in 5-day B.O.D. was 91%.

Acknowledgments

The research reported herein was made possible by funds supplied through a contract with the Wine Advisory Board, state of California.

The helpful advice and cogent criticisms on methods of detartration offered by Mr. Harold W. Berg are gratefully acknowledged.

LITERATURE CITED

1. American Public Health Association. *Standard Methods for the Examination of Water, Sewage and Industrial Wastes.* 10th ed., 1955. Waverly Press, Baltimore, Maryland.
2. Coast Laboratories. Report on grape stillage disposal by intermittent irrigation. Wine Institute, San Francisco, mimeo., 15 pages (1947).
3. Marsh, G. L. Method of determining tartrate and calculation of the amount of precipitants required for its recovery. Wine Institute, San Francisco, mimeo., 5 pages (1942).
4. Marsh, G. L. Recovery of tartrates from winery wastes. Institute of Food Technologists, *Proceedings,* **1943**, 183–195 (1943).
5. Marsh, G. L., and Vaughn, R. H. Slop disposal system based on tartrate recovery. *Wines and Vines,* **25** (6), 15, 17, 28–30, 35 (1944).

6. MATCHETT, J. R., LEGAULT, R. R., NIMMO, C. C., AND NOTTER, G. K. Tartrates from grape wastes. *Ind. Eng. Chem., Ind. Ed.*, **36**, 851–857 (1944). Also see *Wines and Vines*, **24** (11), 23 (1943).
7. PEARSON, E. A., FEUERSTEIN, D. F., AND ONODERA, B. Treatment and utilization of winery wastes. Sanitary Engineering Laboratory, Univ. of California, Berkeley, mimeo., 14 pages (1955).
8. VAUGHN, R. H., AND MARSH, G. L. The disposal of dessert winery wastes, *Wine Review*, **13**, 8–11 (1945).
9. VAUGHN, R. H., AND MARSH, G. L. Disposal of California winery wastes. *Ind. Eng. Chem.*, **45**, 2686–2688 (1953).
10. VAUGHN, R. H., AND MARSH, G. L. Problems in disposal of California winery wastes. *Am. J. Enology*, **6**, 26–34 (1956).

ASEPTIC AUTOLYSIS OF MUSCLE: BIOCHEMICAL AND MICROSCOPIC MODIFICATIONS OCCURRING IN RABBIT AND LAMB MUSCLE DURING ASEPTIC AND ANAEROBIC STORAGE

R. ZENDER, C.-LATASTE-DOROLLE, R. A. COLLET, P. ROWINSKI, AND R. F. MOUTON

Battelle Memorial Institute, Geneva, Switzerland

(Manuscript received August 24, 1957)

In vivo muscle is normally sterile. This tissue becomes highly contaminated post mortem by aerobic and anaerobic flora, however, if it is stored at normal or higher temperature and if it is not protected against contamination from these sources by rather drastic technics.

It has been shown that irradiation can protect muscle from such contamination, but we know that sterilization doses of radiation do not completely inhibit proteolysis *(8)*. We have chosen not to rely on such sterilizations for our first experimentation. We have preferred, rather, to work under strictly aseptic conditions and to excise the muscle material on which we observe modifications during its normal aseptic autolysis.

EXPERIMENTAL

Materials. Lambs (50 kg.) and rabbits (3 kg.) have been used. For practical reasons, we have limited our observations to the dorsal muscles of animals in good health and more particularly those free from Bang's and other diseases. Excision was done as follows:

The animal was first sheared and then thoroughly washed with soap and water. It was epilated with SBa-starch-ZnO mixture on the whole surface of the back and washed again with luke-warm water. It was then slain and bled by severing the throat veins. Thereupon, we laid the animal on an operating table and covered it with sterile towels. The animal, still on the table, was carried into a special room where the atmosphere had been sterilized both by UV rays and Cresol aerosols. (Operators wore sterile blouses, masks and gloves; instruments were heat sterilized). The hide was cut along the spine and turned over revealing a large operating area; this area was bordered by sterile towels. Dorsal muscles thus denuded were excised from the ribs and the apophysis.

Working with the lamb, the muscles were cut under UV rays into pieces of approximately 50 gr.; with rabbits, muscles were stored with no further dissection.

To protect our muscle samples against oxidation by atmospheric oxygen, we put them in large special sterile test tubes composed of two compartments. In the first compartment, we introduce 10 cc. of fresh alkaline pyrogallol solution (25 g. pyrogallol + 75 cc. H_2O + 10 g. NaOH in pellets). In the second one, the muscle sample was introduced immediately after dissection; the tubes were then hermetically closed with rubber stoppers. The samples were thus protected against evaporation.

This method has become routine in our laboratory, and we find that a trained operator can prepare and operate on 5 rabbits per day. For a lamb, we need 3 persons and the operations are somewhat refined with regard to sterile manipulations. Bacteriological controls show that the percentage of contaminated samples excised in this manner is practically nil. However, each sample is tested after storage for the possible presence of aerobic or anaerobic organisms.

We find that the presence of pyrogallol enables us to keep unaltered the original color of our samples. But, if pyrogallol is absent, the samples soon lose their pink pigmentation and become light brown—probably through the formation of met-pigments.

The samples protected in this manner simultaneously against contamination, oxidation, and dehydration were stored in the dark at temperatures ranging from 25 to 37° C. Under

these conditions, it seems highly probable that further modifications occurring in these muscles can be attributed to aseptic autolysis, i.e. to the establishment of rigor mortis and to the action of endocellular proteolytic enzymes (cathepsins).

MACROSCOPIC MODIFICATIONS

Muscles lose all their elasticity after a few hours at normal temperature. The pH of the muscle tissue drops sharply from 7.4 to about 5.6 and, at the beginning of storage, we find that our material is quite hard and tough. These first modifications can be attributed without doubt to the establishment of rigor mortis. In the following period of storage which, in our experimentation, lasted up to 150 days at 25° C. and up to 15 days at 37° C., the rigor relaxes slowly and muscle becomes more and more tender. This tenderization is very probably due to the action of proteolytic enzymes. We can see, however, in Figure 1 that the most important macroscopic modification consists in the production of a liquid exudate which drips from meat during storage.

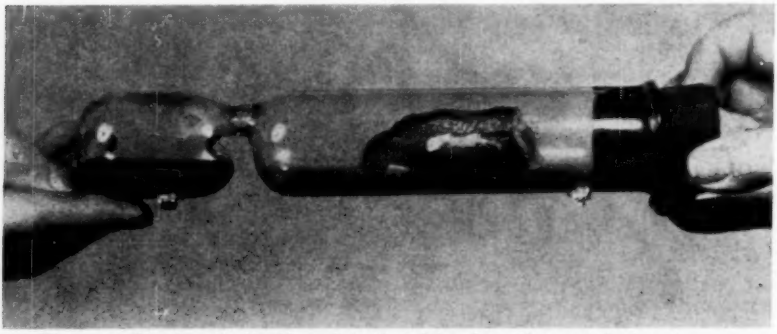


Figure 1. Showing the liquid exudate that drips from the meat during storage.

Using a special tube with an outlet for aseptic collection of exudate at regular intervals, it was possible to measure the amount of exudate during 50 days and to analyse this exudate for the presence of proteins, amino-acids, sodium, and potassium.

Sodium and potassium were measured by flame photometric methods on EEL flame-photometer; amino-acids and proteins were estimated by their tyrosine index using micromethods similar to method C explained in the following section. As shown in Figure 2, exudation begins on the first day of storage and is most important in the first days of storage. This loss in the osmophylic properties of muscle seems to be related to the establishment of rigor mortis. It continues, however, more slowly and, at 51 days storage, the total exudate amounts to 15 to 20% of the original weight of the muscle sample. Figure 2 also shows that the composition of the exudate varies little during storage. This liquid is rich in proteins (7 g. %, approximately) and potassium (0.4 g. %). It is poor in sodium and amino-acids (i.e., compounds not precipitated by trichloracetic acid, 10%).

The presence of large amounts of potassium in the exudate during the first days of storage shows clearly that the osmotic properties of cellular memberings have been lost. It points also to the intracellular origin of this exudate.

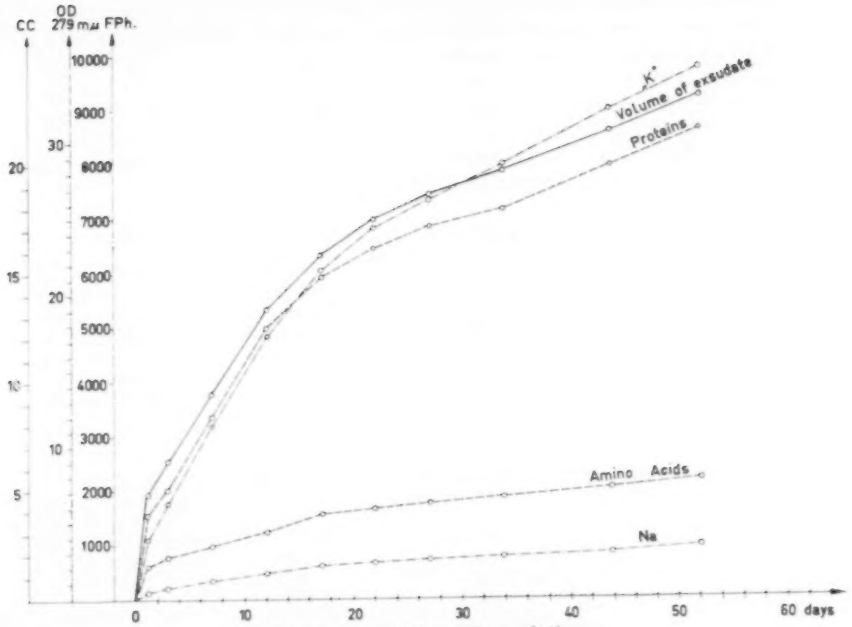


Figure 2. Rabbit muscle exudation.

Further histological modifications occurring at the same period of storage re-enforce this assumption.

In fact, the very large amount of proteins contained in the exudate excludes the possibility of this being an extracellular fluid. We know that interstitial liquid is very poor in proteins. These proteins of the exudate are characterized by their solubility in solvents of relatively low ionic strength (KCl 0.1 M). However, 10% of them precipitate if the exudate is diluted 25 times with distilled water.

Proteins of the muscle can be listed in 4 different groups, defined, following their solubility and their physiological properties, as in Table 1.

TABLE 1
Muscle Proteins

Group	Protein	Solubility
Myogen proteins, 28%	Myoalbumine, Globuline x Enzymes, etc.	Distilled water or salts 0.05–0.1 M
Muscular plasma proteins, 52%	Myosin, Actin, Tropomyosin, etc.	Salts 0.3–0.6 M glycin pH 8, 6
Stroma proteins, 16%	Collagen, etc.	Weak acids, bases or insoluble
Others		Insoluble

We may conclude from Table 1 that the proteins of the exudate belong to the first group containing myoalbumine, etc.; the other proteins of the muscular plasma cannot be soluble in the exudate. Moreover, the 10% proteins which precipitate from the exudate at lower salt concentration could well be globuline X which is known to precipitate under the same conditions. It might be, however, that in later periods of storage other proteins than those of the myogen group come into solution. These would be formed by partial proteolysis of proteins belonging to other groups.

MODIFICATIONS OCCURRING IN THE PROTIDE DISTRIBUTION OF THE MUSCLE

Methods. Muscle proteins, excepting those of the myogen group, require especially powerful solvents for their extraction. KCl 0.6 M is one such solvent, but, as we wished also to study the electrophoretic distribution of the extracted proteins, we were led to choose another solvent which has a lower electric conductivity. In Table 2, we have compared the extractive power on muscle of different solvents in such concentrations that the total amount of proteins submitted to the extraction ranges 0.05 g./100 cc. solvent.

TABLE 2

Dissolving power of different solvents used on muscle proteins

(P)	Solvents	pH	Optical density 279 mμ
↑	Distilled water	5.96	0.107
	Glycine 0.05 M	8.28	0.326
	Glycine 0.1 M	8.42	0.393
	Glycine 0.5 M	8.6	0.268
	Glycine 1.0 M	8.6	0.223
0.05%	KCl 0.05 M	5.95	0.180
range	KCl 0.1 M	5.98	0.153
	KCl 0.2 M	5.94	0.174
	KCl 0.6 M	5.94	0.279
	KCl 1.0 M	6.	0.236
↓	Acetic acid 0.01	3.35	0.277

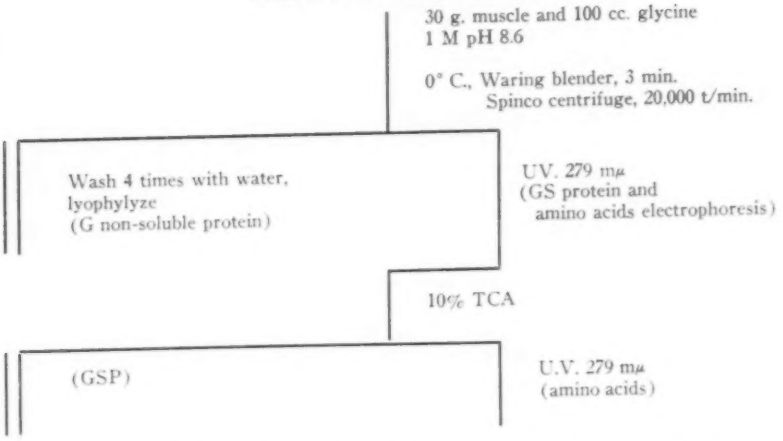
We have found that glycine NaOH buffer at pH 8.4 to 8.6 can be compared favorably with classical solvents for the extraction of muscle proteins. Moreover, this solvent does not denature the extracted proteins and its low conductivity enables us to use it immediately after extraction for electrophoretic analysis. Data (not reported in the present paper) show that if we increase the amount of proteins extracted with a given volume of buffer, the concentration in glycine of the buffer must also be increased. For electrophoretic analysis, we need to have the protein concentration ranging from 1 to 2 g. % and consequently we have used glycine buffer 1 M, pH 8.6. Our method of extraction and analysis is as follows:

Muscle is extracted with three times its weight of glycine buffer 1 M in the Waring blender at 0° C.; the resulting suspension can be very viscous and must be centrifuged at 20,000 r.p.m., for 30 minutes. The clear supernatant is our extract; it contains all aqua-soluble components of muscle and a good part of its fibrillar proteins.

The extract is diluted and its optical density measured at 279 mμ with the Beckman DU spectrophotometer both before and after precipitation of proteins with trichloracetic acid 10%. We know that this 279 mμ wave length is characteristic for tyrosine tryptophane either free or bound to proteins and we have further compared variations in the 279 mμ index with variations in either protein or nonprotein nitrogen concentration. We have thus verified that the variations correspond well one to the other. However, the 279 mμ index must be considered as a relative measure of protide concentration. It is very useful for estimating the level of proteolysis during storage and more particularly in

TABLE 3

EXTRACTION METHOD C



solvents such as glycine 1 M, containing high concentrations of nonprotein nitrogen. Electrophoretic measurements were made at 4° C., 10 mA and during 10,000 sec.

RESULTS

In Figure 3, we have drawn the evolution of tyrosine index during 70 days' storage for both proteins and amino-acids extracted from muscles stored in the standard conditions at 25° C. and 38° C. for lamb and rabbit.

After one day's storage, i.e., after the establishment of rigor mortis, there is registered an important increase in the level of protein soluble in glycine 1 M. This increase was quite unexpected. In fact, in the usual solvents—for instance in KCl 0.6 M—the establishment of rigor mortis corresponds to a diminution of fibrillar protein solubility. Thus, our buffer has this supplementary quality that it permits, after the establishment of rigor mortis, the continuation of the dissolving activity—i.e., the fibrillar proteins of the muscle, more particularly perhaps the actomyosine, continue to solubilize. In the next days and weeks of storage, glycine soluble protein concentration decreases slowly as, conversely, concentration in amino-acids rises. We see in Figure 3 that both these phenomena occur much more rapidly at 38° C. than at 25° C. In fact, we find that the level of amino-acids after 15 days storage at 38° C. is equivalent to the level we obtain only after 100 days at 25° C. Nevertheless, these proteolytic phenomena occur slowly.

With reference to the work done on the autolysis of liver and other tissues, we hoped to observe a more rapid occurrence of the phenomenon, the appearance within the first few days or even in the first few hours of storage of an important increase in amino-acids concentration. This does not seem to happen, at least during the first 31 days of storage at 25° C. This observation has been verified repeatedly on lamb and on rabbit, measurements being made following the tyrosine index or the nonprotein nitrogen level.

In Table 4 we have a comparison of different tissues with reference to

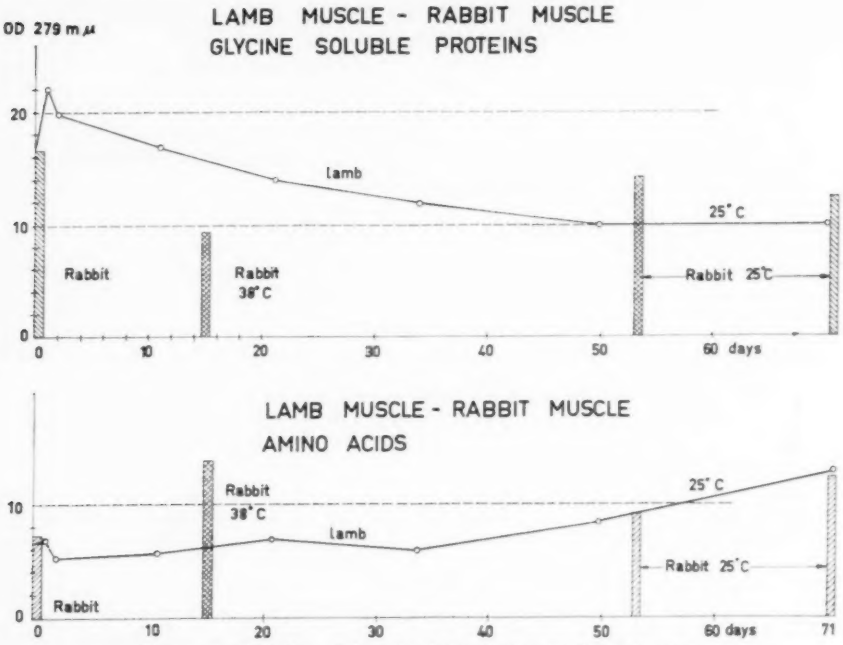


Figure 3. Evolution of tyrosine index during 70 days' storage.

their proteolytic activity. Following variation in the level of free tyrosin tryptophane, we see that the proteolytic activity of muscle, compared to the activity of liver, kidney or lung, is very low.

After 6 days' storage and 42 days' storage, the glycine extract of lamb muscle was submitted to electrophoretic analysis (Figure 4).

On the first day of storage, our electrophoretic pattern is similar to those obtained by other authors, more particularly by J. J. C. Jacob *(19)*. It appears that the 3 different groups of proteins mentioned by this author are present in our extracts. However, owing to the relatively low conductivity of the glycine buffer, we have obtained separations of the different groups of com-

TABLE 4

Compared proteolytic activity of different tissues. 10 g. of tissue in 100 cc. KCl 2%

Tissue	Activity[1]
Kidney	1.900
Liver	1.500
Lung	1.000
Heart	0.330
Psoas muscle	0.025
1/2 kidney + 1/2 muscle	1.100
1/2 liver + 1/2 muscle	0.930
1/2 lung + 1/2 muscle	0.608

[1] Expressed as increase in optical density at 279 mμ.

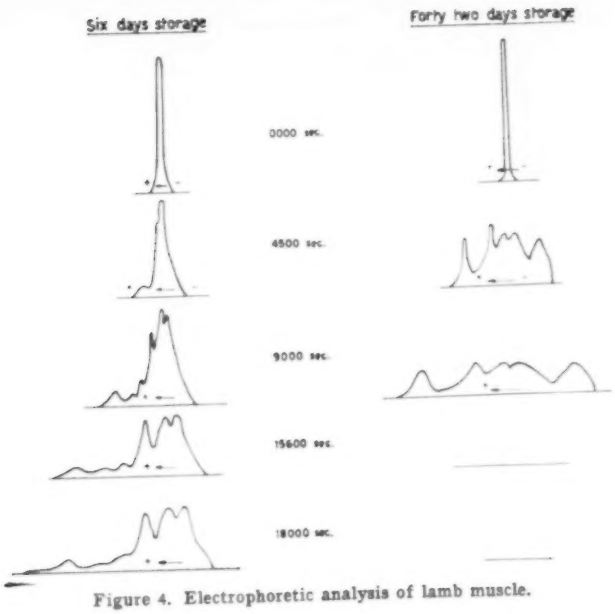


Figure 4. Electrophoretic analysis of lamb muscle.

ponents, with smaller duration of electrophoresis. It was not in our present program to analyse the different electrophoretic fractions that we have obtained, but it might be most interesting to use the glycine buffers and measure the electrophoretic mobilities of different muscle constituents, principally myosine, actine, etc.

A run of 18,000 sec. at 10 mA shows that the major part of the extracted proteins are characterized by low mobilities at pH 8.6. On the contrary, after 42 days of storage, we see that the electrophoretic distribution of the exrtacted proteins has been greatly modified. In fact, we observe full resolution of the electrophoretic diagram after 9,000 sec. and after 4,500 sec., the peaks are already very well separated. Systematic electrophoretic analyses have been done at different intervals during 100 days' storage at 25° C. These observations have shown that the electrophoretic diagram for extracted proteins is modified from the 20th day of storage on. Rabbit muscle behaves in a similar manner with this difference that its electrophoretic diagram is only modified after 50 or 70 days' storage in aseptic and anaerobic conditions.

On the other hand, if rabbit muscle is stored at 38° C., the electrophoretic modifications occur more rapidly, as shown in Figure 5:

In fact in Figure 5, we see clearly after 9 days that new protidic constituents have appeared and that after 15 days they amount to about one half of the total proteins of the extract if we compare surfaces.

For the present, until further statistical measurements have been made, we shall not take into account in these diagrams modifications of lesser importance which occur during storage and we also do not wish to take into

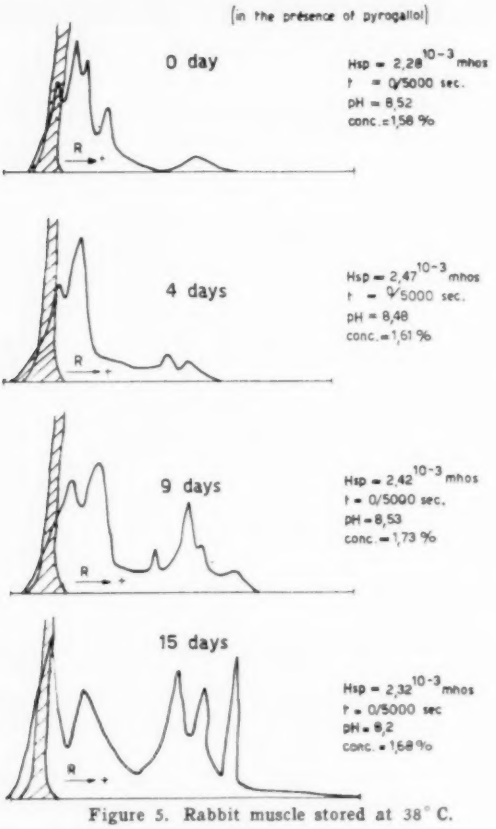


Figure 5. Rabbit muscle stored at 38° C.

account small differences existing between diagrams and to measure their exact relative importance. This work is actually in progress, however, and will be reported later. In our present interpretation, we shall only insist on the massive and reproducible appearance of new proteic constituents in muscle, these constituents being characterized by their higher electrophoretic mobility.

We have tried to reproduce proteolytic phenomena occurring during storage. For this, we have treated muscle or its extracts with highly active proteolytic enzymes such as papain or trypsin.

We have shown thus that the first effect of papain or trypsin on the muscle consists in the liberation of glycine soluble proteins. On the other hand, amino-acids do not increase in the first stage of papain or trypsin action. This model of the proteolysis of muscular tissue shows that the fibrillar proteins of the muscle in their native state are relatively resistant to the action of proteolytic enzymes, and are not split in this stage into amino-acids or fragments of low molecular weight, soluble in trichloracetic acid. However, it

TABLE 5

Lamb muscle: protide variations in the presence of papain and trypsin

	Proteins Optical density 279 mμ	Amino-acids Optical density 279 mμ
Without papain	10.75	6.25
With papain	23.6	6.25
Without trypsin	11.00	6.40
With trypsin	19.7	7.26

seems that they are modified and rendered more soluble by the action of these enzymes (Figure 6).

The action of papain has not modified in an important manner the electrophoretic pattern of the GSP. The action of papain might be thus limited to the liberation of soluble proteins (Figure 7).

On the contrary, trypsin induces deep modifications in the electrophoretic pattern of the GSP and seems to act in the same manner as do endocellular proteolytic enzymes during storage. These observations and more particularly those done on papain might explain the action of enzymatic commercial preparations which promote tenderization of tough meat samples. The action of these products would consist of a limited digestion of fibrillar or perhaps connective proteins of the muscle.

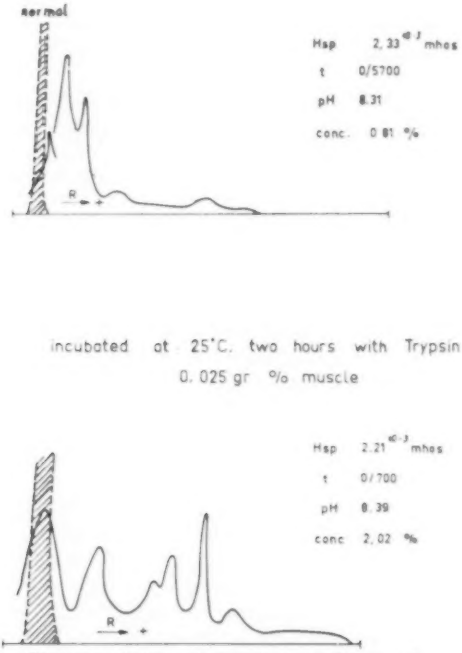


Figure 6. Action of trypsin on rabbit muscle.

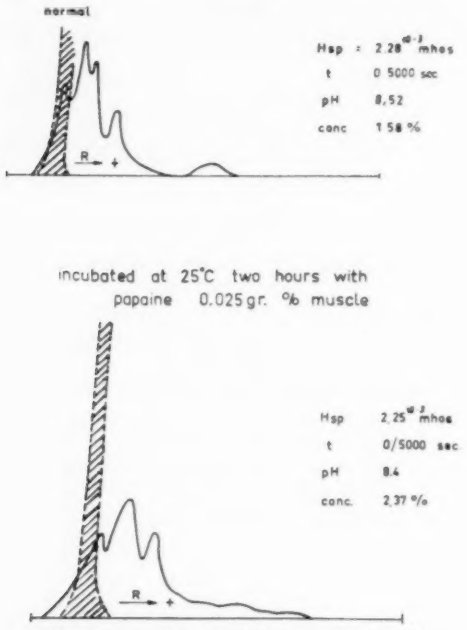


Figure 7. Action of papain on rabbit muscle.

MICROSCOPIC STRUCTURAL MODIFICATIONS

Physico-chemical modifications observed in muscle during aseptic and anaerobic storage have been correlated with modifications occurring in the structure of the muscular fibre. These modifications have been observed both at the fibre and the myofibrils level.

The systematic study by interference microscopy, in positive contrast illumination, shows plainly the classical cross-striated appearance of voluntary muscle fibres: succession of alternating "anisotropic" (high dense, birefringent) and "isotropic" (low dense, less birefringent) bands.

The myofibrils display the same band-pattern as the fibres in which they are packed in such a way that the respective striations are held in register and give the appearance of continuous rings across the whole width of the fibre *(17)*.

It has been suggested that the contractile material, contractile proteins, myosine and actine essentially, is contained in the myofibrils *(12-15)*. The concept of the localisation of myosin in the anisotropic band has been placed very recently on an experimental basis, together with interference microscopy and biochemical techniques *(13, 18)*. These important data give value to the theoretical structure model *(14)* in which two sets of overlapping filaments, actin and myosin filaments, slide past each other as the muscle changes its length in vital contraction and stretching as well as in rigor mortis contraction.

Undoubtedly, a new morphological feature is the expression of a molecular change.

Combined with microphotography, the morphological investigation in interference microscopy is accordingly a good practical way to help in testing, during storage, intimate structure modulus and biochemical correlations.

MATERIAL AND METHODS

A small sample of muscle, stored in the standard aseptic anaerobic conditions previously described, has been respectively dissected the 1st, (ante and post rigor mortis), 2nd, 3rd, 10th, 20th, 40th, 50th, 70th and 100th day.

The fibres are dissected in the mixture that we call the "SGH" solution:

—one part of Szent-György 50% glycerol *(10)*,
—one part of Hodge solution KCl 1 M – $MgCl_2$ · 0.001 M

The dissection is carried out under a stereoscopic magnifier (magnifying x 50 to x 100), directly on the coverslip, with very thin anatomic needles (commonly used to guide biopsies). Fibres are thus separated with less damage. The coverslip is turned over on a special slide coated with gelatin. Being hygroscopic, gelatin attracts the preparation, making a soft settling *(20)*. The fibres can thus be maintained for quite a long period of observation without apparent physical deterioration.

The positive phase contrast illumination darkens the anisotropic bands and lightens the isotropic ones; the negative phase contrast acts oppositely.

Various magnifications are used for direct examination: x 250, x 500 and even x 800 to x 1000 (objective numeral apertures: 0.60 – 0.75 and 1.30 condenser aperture: 0.6). Examination is made on several gelatin slides for each sample. Chosen fields containing suitable selected fibres are photographed both in topographic and detailed views (x 200 to x 500 magnifyings). Further enlargements are carried out on diapositives or papers.

This method of investigation enables us to describe and classify some of the principal changes:

—in time of dissection, anatomic aspect is appreciated: consistency and hardness of the meat, extensibility of the fibre;
—microscopically, the changes concern mainly the modification of the apparent walls in the sarcolemna and the transversal striations referred to above.

The modifications on the walls are principally the "flexuring" and the "shrinking" (feature of accordion in acute flexuring), transversal "splitting" in site of flexuring (aspect of "chess-board" in complete splitting), longitudinal splitting (special aspect of myofibrils fan).

The modifications inside the fibre alter the striation which are more or less degenerated from the normal straight transversal bands to the "hyphened" lines (– – – – – – – dotted lines) and to the granular stage (. . . . . : scattered spots).

Microscopically, we are able to appreciate turgescency, flatness, and flabbiness.

The last stage of degeneration is characterized by areas of residual muscular material in which no individual fibre can still be recognized. These areas are superficially appreciated.

RESULTS

During 100 days of storage, the principal modifications and special features observed were the following:

A. Anatomically.

The tender smooth and extensible fibre of the fresh sample of the first day, before rigor, stiffens and hardens in the first few hours. It loses its extensibility.

After 10 and 20 days' storage, dissection becomes easier to perform than just post rigor. Partial resolution of the rigor has occurred but the fibre remains very less extensible than ante rigor.

Between the 40th and the 50th day, it is difficult to separate long intact fibres and between the 70th and 100th day, the meat becomes more and more tender until it disintegrates by itself as soon as it is put in SGH solution (100th day).

B. Microscopically.

1. *Changes in the wall.* Rigor mortis induces the formation of indentations in the walls during the first 3 days (Figure 8). Some waving of the whole fibre may appear at this stage.

Between the 10th and the 20th day, flexuring of the fibre occurs more or less sharply. The beautiful feature, the "accordion," is often encountered (Figure 9) with sometimes the beginning of disruptions in site of the plaits. We consider flexuring and this partial disruption as the first morphological data related to exudative phenomena.

The main feature observable between the 40th and the 50th day is the general transversal splitting which ends in the "chess-board" stage, characterized by flat squares (c.f. Figure 13 "stair-steps" and "chess-board"). At this stage, general disruption and flatness is probably related to strong exudation and modifies completely in a kind of "turn-over" the former balance of straight and flexured fibres (cf. graph, Figure 16).

From the 50th to the 70th day and until the 100th day areas of complete degeneracy increase progressively from nearly 1:10 up to 1:2. The special aspect, "myofibrils fans" (Figure 14), is produced on still recognizable fibres by longitudinal splitting. We consider this feature as a step of the progressive alterations of connective tissue between the myofibrils.

Figure 8. Two days' storage: "rigor contraction," indented walls, well kept band pattern.

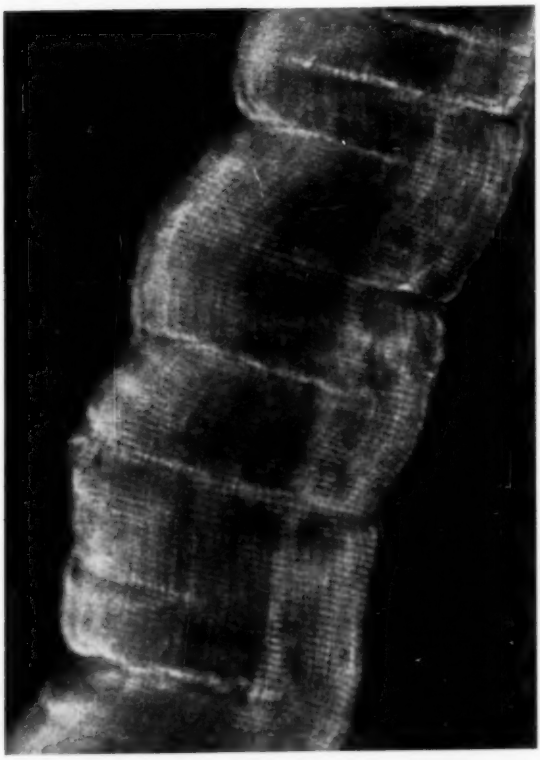


Figure 9. Ten days' storage: flexuring, "accordion."

2. *Changes in striations.* The classical band pattern of fresh fibres remains intact in rigor contraction during the first 2 or 3 days.

On the 10th day, new aspects of "hyphened" and even "granular" (Figure 10 and 11) bands appear inside the fibre. Numeration in percentages of normal and altered striations are summed up in Table 6 and on the graph (Figure 17).

Between the 20th and 50th day, the rate of degeneracy is different if we compare straight and flexured fibres.

On the 50th day, degeneracy inside the two kinds of fibres is equivalent.

On the 70th day, 5% only and on the 100th day 1 to 2% of the original band pattern can still be recorded. At that time rare bundles of isolated myofibrils still show clearly the normal band pattern.

A special feature appears on the residual well kept fibres; it consists of a kind of stretching between the anisotropic bands showing the Z-band clearly with magnifications X800, X1,000. We believe that this new structural aspect on a fibre stored for 100 days can be related to more severe alterations of the molecular structure.

C. Graphically.

By means of the following pictures, chosen from among some 200, we have tried to show the main characteristic aspects and changes which occur during 100 days' storage.

TABLE 6

Normal and altered striations

	Muscle fibres								Areas of degeneracy (2)	Observations in time of dissection and main features under phase contrast	Reference plate
	Straight fibres				Flexured fibres						
	Total	(1)	(1)	(1)	Total	(1)	(1)	(1)			
0 day	100	100	....	....	0	....	....	....	....	*Elasticity*—Turgescency	
										—linear walls	1/0
1–2 days	90	100	....	....	10	....	....	....	....	*Stiffening*—Turgescency	
										—indented walls	10/2
10 days	72	56	40	4	28	80	20	....	....	*Flexuring*—Turgescency	
										—topographic area	12/10
										—aspect of "accordion"	10/10
										—aspect of "hyphened band"	3/10
										—aspect of granular band	11/10
20 days	65	40	44	16	35	62	36	2	1 : 20	*Flexuring*—loss of turgescency	
										Outset of transversal splitting	
										—splitting	12/20
										—splitting	15/20
										—degeneracy	7/20
40 days	60	26	38	36	40	55	43	2	1 : 10	*Flexuring-Flattening*	
										Complete transversal splitting	
										—aspect of square puzzle "chess board"	4/40
50 days	80	10	46	44	20	3	57	40	1 : 5	*Longitudinal splitting*	
										—fan shaped myofibrils	4/50
										—undamaged and damaged fibres + degeneracy	3/50
70 days	89	5	43	52	11	13	47	66	1 : 3	*Tenderness*	
										—"chess board" in degeneracy	3/70
										—twisted fibre	11/70
100 days	94	2	37	61	6	1	19	80	1 : 2	*"Flabbiness"-"Stretching"*	
										—disruption/Z band	7/100
										—well kept myofibrils	9/100

1) Main features in striations: continuous, hyphen and granular bands.
2) No visible sarcolemna.

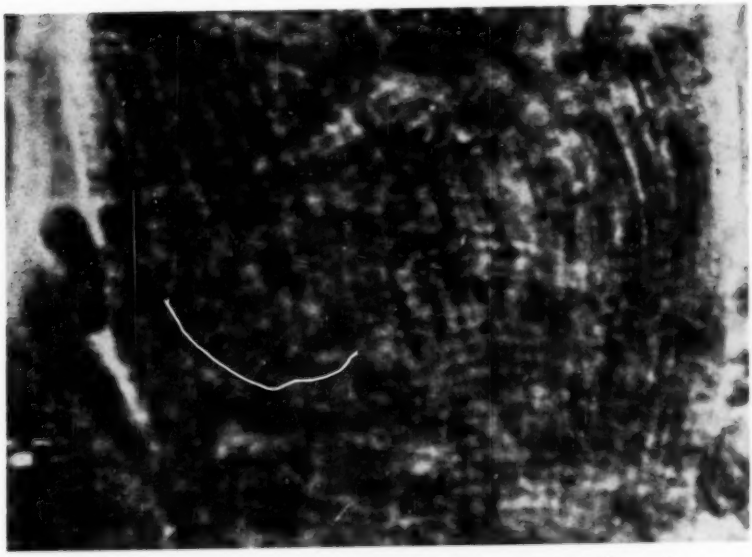


Figure 10. Ten days' storage: damaged striation, "hyphened" aspect.

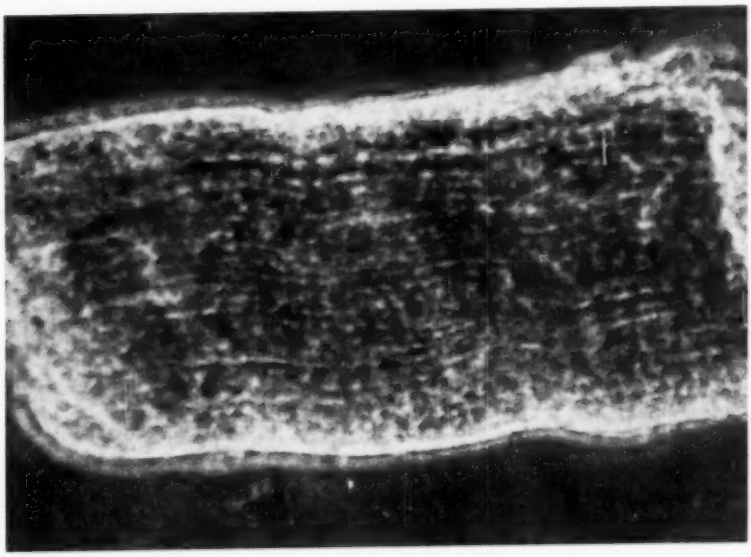


Figure 11. Ten days' storage: damaged striation, "granular" aspect.

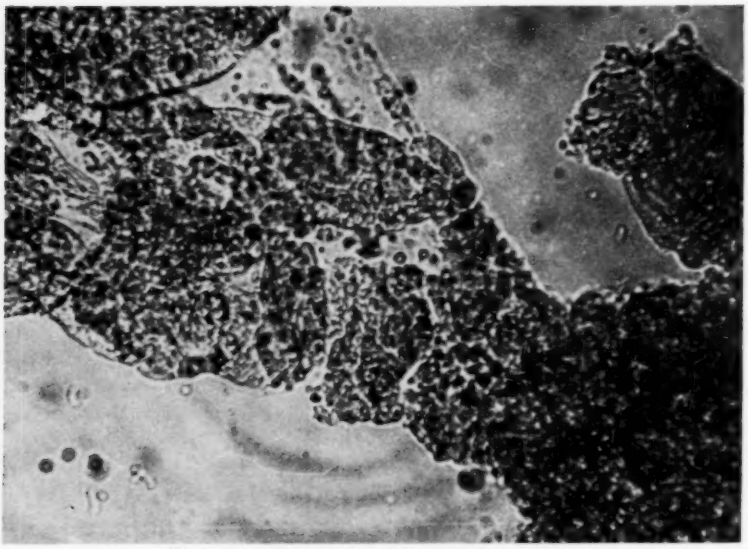


Figure 12. Twenty days' storage, degeneracy.

DISCUSSION

Results presented in this paper must be considered as preliminary and conclusions we might be tempted to make should remain quite general. It is however obvious that the modifications observed in our aseptic samples will have a definite influence on the storage qualities of radiation sterilized meat.

The most important phenomenon, as mentioned above, which occurs during sterile autolysis, is exudation. This exudation consists in an important loss of water, proteins and potassium *(16)*. We think that this exudation results priorily from the establishment of rigor mortis and from the parallel lowering of the pH of the muscle. We do not think that it is directly related to proteolytic phenomena. Structural microscopic observations enable us to relate also this exudation to the flexuring and loss of turgescency of the fibres. It can also be related to the accordion aspect. Muscular tissue degradation can be separated in two stages: the first stage lasts only a few hours and consists in the establishment of rigor mortis. Enzymes which are responsible for this stage are the classical enzymes of glycolysis, which catabolize glycogen into lactic acid in the absence of oxygen. We find during this stage a concomitant loss of energy rich phosphate groups, particularly of adenosine triphosphate (ATP) *(2)*. At the end of the first phase, muscle behaves like a rigid tissue with acid pH (5.5 to 6.0). It has lost the potential energy necessary for contraction. At the level of fibrillar proteins, deep modifications have occurred, myosine and actine have reacted to form actomyosine. This actomyosine is not further split as happens in vivo into myosine and actine. We must admit that after death, the formation of actomyosine creates in the fibrils a longitudinal tension. This tension explains the formation of flexured and accordion fibres. It might also explain the production of exudate and a concomitant loss of

Figure 13. Forty days' storage: transversal splitting, "chess-board" and "stair-steps."

turgescency. As exudation is relatively slow and as the production of accordion fibres depends also on the loss of the elasticity of fibrillar memberings, the development of the first phase of degradation will last 1 or 2 weeks.

However, we may consider that the second phase of muscular degradation begins immediately after the establishment of rigor mortis and lasts until the complete autolysis of muscular tissue. Until now, we have observed this autolysis for 150 days at 25° C. The enzymes responsible for this second stage are the cathepsines of the muscle *(7, 24, 25)*, their optimum proteolytic activity is probably located as for other tissues between pH 4 and 6 *(1, 3, 4, 6, 11, 22, 26)*. Their activity at pH 7.4 (pH of muscle in vivo) is practically nul. It is thus evident that the second stage of muscular degradation can begin only after the establishment of rigor mortis, i.e. when the pH has become acid.

If we compare muscle to other tissues, we find that the cathepsines of muscle do not liberate in the first hours of autolysis, free amino-acids or polypeptides not precipitated by trichloracetic acid; we do not know exactly if this must be attributed to the low concentration of the cathepsines in muscle or on the contrary to the resistance of the substrate, i.e. muscular proteins to the action of proteolytic enzymes in general.

We would be tempted to accept the first hypothesis, which says proteolytic

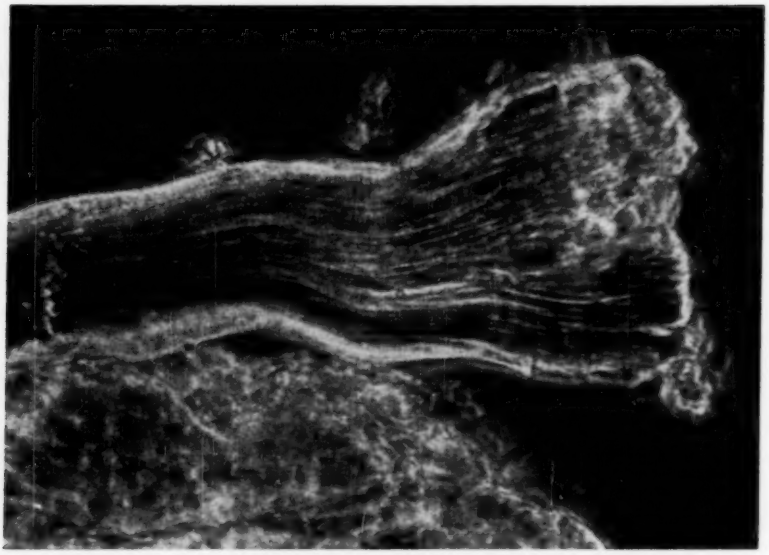


Figure 14. Fifty days' storage: longitudinal splitting, "myofibrils fan."

enzymes exist only in very low concentrations in muscle, as we have verified that the liberation of amino-acids is not enhanced by the addition to muscular extracts of a substrate like hemoglobin which is easily split by proteolytic enzymes. On the other hand, we cannot neglect the second hypothesis, as we have seen that purified and highly active proteolytic enzymes cannot split, in the first phase of their action at least, the proteins of muscle into compounds of low molecular weight. Thus we must think that autolysis in muscle is characterized both by the presence of proteolytic enzymes at very low concentration, and by a substrate particularly resistant to the action of these enzymes.

However the action of proteolytic enzymes can be observed in muscle also by other methods. We have seen in fact that, from the 21st day of sterile storage on, the electrophoretic diagram of muscular proteins extracted in our glycine buffer shows that new components have appeared. These new proteic constituents of muscle in storage are more and more clearly observed during storage at 25 or 38° C.

The exact signification of these new electrophoretic diagrams is not entirely known. One could suggest that the action of muscle cathepsine is limited, at least at the beginning of storage, to partial hydrolysis of high molecular weight fibrillar proteins into proteic subunits of lower molecular weight. These new proteins seem to be characterized by higher electrophoretic mobilities. Later, during storage, free amino-acids are liberated in increasing amount. These observations confirm those stated by Szent-Györgyi and other authors *(3, 21, 23, 27)* who have studied the action of purified trypsin or of alkaline urea denaturation on solutions of purified myosine. Szent-Györgyi has demonstrated more particularly that myosine was split into smaller molecules, meromyosine H and L.

Figure 15. One hundred days' storage: some myofibrils at 100th day, well kept band pattern.

Interference microscopy shows us that the second phase of degradation is characterized first and until the 30th day by the phenomena of flexuring, phenomena that we have attributed to longitudinal tension in the fibres and to the progressive loss of intracellular fluid after the establishment of rigor mortis. Proteolytic phenomena revealed by modification of the electrophoretic diagram and liberation of free amino-acids can also be seen under the microscope. Transversal splitting of the fibres, more particularly located where flexuring has occurred, disruption of these fibres and formation of "chessboard," longitudinal splitting and, finally, degeneracy of the internal structure of the fibres, disappearance of the band pattern replaced by granulation pattern, all these aspects and other like myofibrils "fans," are characteristic of the second stage. We have seen that these phenomena are more and more important in number during storage. However, we have also seen that a very small percentage of fibres can keep their intact structure even until 70 days' storage at 25° C. These phenomena should be observed more closely at the level of the myofibrils with new histo-chemical techniques *(5)* in which muscular proteins can be removed from the bands on the slides under microscope, and possibly with electron microscope at the level of the A and I bands. It seems however clear that if we can relate rigor mortis to the phenomena of

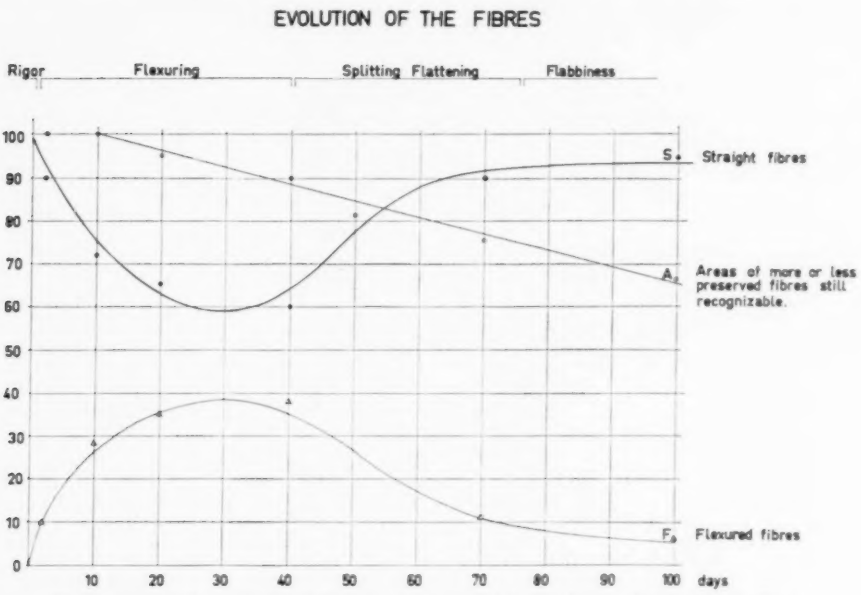


Figure 16. Mutton muscle (25° C. aseptic anaerobic) showing evolution of the fibre.

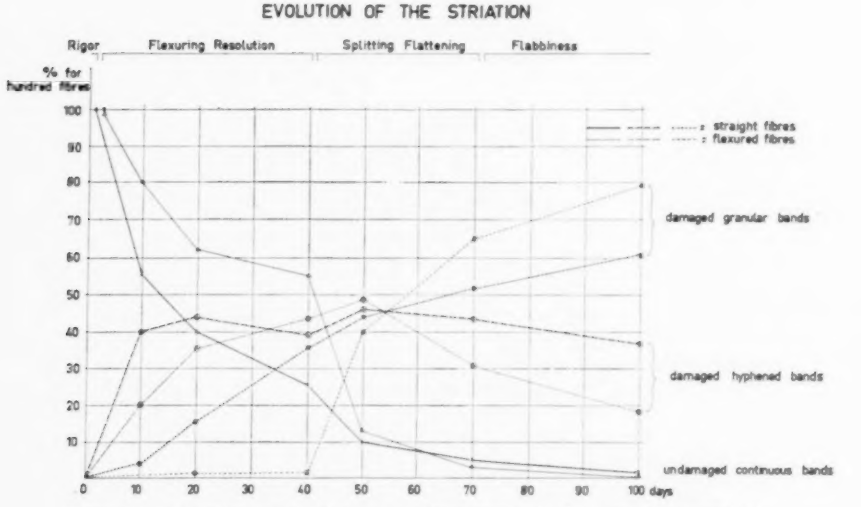


Figure 17. Evolution of striation of mutton muscle (25° C. aseptic anaerobic).

flexuring, turgescency and exudation, on the one hand, on the other, proteolytic phenomena are to be related to the progressive appearance of cellular degeneracy unto the loss of all the structural characteristics of the fibre.

The results reported here on muscle autolysis will, in future work, be related to meat processing technology. We will, in particular, study the effect of ionizing radiations. At sterilizing doses (1 million to 4 millions rad) ionizing radiations are known to destroy a certain amount of enzymes. This enzymatic inhibition by irradiation must be studied measuring the influence of sterilizing doses, both by beta and gamma rays, on the autolytic phenomena described in this paper.

SUMMARY

The natural aseptic and anaerobic degradation of muscle has been followed for 150 days at 25° C. and for 15 days at 38° C. The authors have observed a slow rise in the level of amino-acids and a parallel decrease of glycine soluble proteins. The electrophoretic distribution of muscle proteins is modified during storage as if proteins were first split into subunits and only later into amino-acids. The exudate produced during 50 days storage amounts to 15% of the original muscle samples, it contains around 7 g. % of proteins and 0.4 g. % of potassium. A method is described to follow structural modifications by interference microscopy. Different stages of autolysis are defined and modifications in the microscopic structure are related to the biochemical and macroscopic evolution of muscle properties and constituents.

Acknowledgment

Acknowledgment is gratefully made to the General Electric Company, in Schenectady, who generously sponsored this research.

The authors wish also to acknowledge the suggestions made by Dr. W. D. Bellamy of the General Electric Research Laboratory, and by Dr. P. E. Després of the Geneva Slaughter Houses.

The authors wish to express their gratitude to those who have aided in this research. These include R. Prince, J. Messi and D. Larue.

LITERATURE CITED

1. Anson, M. L. The purification of cathepsine. *J. Gen. Physiol.*, **23**, 1, 695, (1939).
2. Bate-Smith, E. C. Changes in meat accompanying rigor mortis. *Adv. in Food Res.*, 1 (1948).
3. Dannenberg, A. M., Jr., and Smith, E. L. Proteolytic enzymes of lung. *J. Biol. Chem.*, **215**, 45 (1955).
4. Dannenberg, A. M., Jr., and Smith, E. L. Action of proteinase. I. Bovine lung. Hydrolysis of the oxidized B chain of insulin; polymer formation from amino-acid esters. *J. Biol. Chem.*, **215**, 55 (1955).
5. Davies, H. G., and Deeley, E. M. An integrater for measuring the "dry mass" of cells and isolated components. *Exptl. Cell Research*, 11, 169 (1956).
6. Dianzani, M. U. The nature and mechanism of action of the enzymes responsible for tissue respiration during aseptic autolysis. *Biochem. J.*, **49**, 427 (1951).
7. Doty, E. M., and Wachter, J. P. Influence of gamma-radiation on proteolytic enzyme activity of beef muscle. *J. Agr. Food Chem.*, **3**, 1, 63, (1955).
8. Drake, M. P., Giffee, J. W., Jr., Ryer, Robert, III, Harriman, Hubert. Proteolytic enzyme activity in irradiation-sterilized meat. *Science*, **125**, 23 (1957).
9. Gercely, J. Studies on myosin-adenosinetriphosphatase. *J. Biol. Chem.*, **200**, 543 (1953).

10. GUBA AND SZENT-GYÖRGYI. *Chemistry of Muscular Contraction.* Academic Press, N. Y.
11. GUTMANN, H. R., AND FRUTON, J. S. On the proteolytic enzymes of animal tissues. VIII. An intracellular enzyme related to chymotrypsin. *J. Biol. Chem.*, **215**, 163 (1955).
12. HANSON, J., AND HUXLEY, H. E. Structural basis of the cross-striations in muscle. *Nature*, **172**, 530 (1953).
13. HANSON, J., AND HUXLEY, H. E. Quantitative studies on the structure of cross-striated myofibrils. II. Investigations by biochemical techniques. *Biochim. et Biophys. Acta*, **23**, 250–260 (1957).
14. HANSON, J., AND HUXLEY, H. E. *Symposia Soc. Exper. Biol.*, **9**, 228 (1955).
15. HASELBACH, W. Elektronenmikroskopische Untersuchungen an Muskelfibrillen bei totaler und partieller Extraktion des L-Myosins. *Z. Naturforsch.*, **86**, 449 (1943).
16. HENRY, M., BILLON, J., AND HAONZA, G. Contribution à l'Etude de l'Acidose des Viandes du Porc, dites exsudatives. *Revue de Path. générale et comparée*, **669**, 857 (1955).
17. HUXLEY, A. F. Applications of interference microscope. *J. Physiol.*, **117**, 52 P (1952).
18. HUXLEY, H. E., AND HANSON, S. Quantitative studies on the structure of cross-striated myofibrils. I. Investigations by interference microscopy. *Biochim. et Biophys. Acta*, **23**, 229–249 (1957).
19. JACOB, J. J. C. The electrophoretic analysis of protein extracts from striated rabbit muscle. *Biochem. J.*, **41**, 83-94 (1947).
20. KELLENBERGER. Personal techniques, Biophysic, Geneva.
21. MIHALYI, E., AND SZENT-GYÖRGYI, A. G. Trypsin digestion of muscle proteins. I. Ultracentrifugal analysis of the process. *J. Biol. Chem.*, **201**, 189 (1953).
22. MORRISON, M. L., AND NEURATH, H. Proteolytic enzymes of the formed elements of human blood. *J. Biol. Chem.*, **200**, 39 (1953).
23. PERRY, S. V. The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle. *Biochem. J.*, **48**, 257 (1951).
24. SMITH, E. L. The peptidases of skeletal, heart, and uterine muscle. *J. Biol. Chem.*, **173**, 1, 533, (1948).
25. SNOKE, J. E., AND NEURATH, H. The proteolytic activity of striated rabbit muscle. *J. Biol. Chem.*, **187**, 1, 127, (1950).
26. TALLAN, H. H., JONES, M. E., AND FRUTON, J. S. On the proteolytic enzymes of animal tissues. X. Beef spleen cathepsin. *J. Biol. Chem.*, **194**, 793 (1952).
27. TSAO, T. C. Fragmentation of myosine molecule. *Biochim. et Biophys. Acta*, 11, 368 (1953).

NOTES AND LETTERS

VITAMIN B_6 IN CANNED PINEAPPLE PRODUCTS*

Vitamin B_6 values for canned sliced pineapple and canned pineapple juice were reported as part of a survey on canned foods in 1953 *(4)*. The levels in pineapple juice were higher than those in sliced pineapple (total contents of the can). Recent information on canned *(2)* and frozen *(1)* pineapple products suggested that the values reported for pineapple juice in the 1953 publication might not be typical for that product. Consequently new samples were obtained from 5 different producers of canned pineapple juice and sliced pineapple in the Hawaii Islands. Samples for vitamin B_6 assay were prepared by compositing 6 cans of each sample. Aliquots were autoclaved for 2 hours at 15 lbs. in 0.06 N H_2SO_4. A microbiological assay procedure *(3)* was employed.

The following results were obtained (expressed in mg. per 100 g.):

Canned sliced pineapple		*Canned pineapple juice*	
Range	0.061 — 0.082	Range	0.080 — 0.125
Average	0.071	Average	0.096

The average values reported in 1953 were 0.077 for canned sliced pineapple and 0.33 for canned pineapple juice. It appears that the latter value is high and not typical of the product.

L. J. TEPLY
Wisconsin Alumni Research Foundation
506 N. Walnut
Madison, Wisconsin

January 9, 1958

LITERATURE CITED

1. BURGER, M., HEIN, L. W., TEPLY, L. J., DERSE, P. H., AND KRIEGER, C. H., *J. Agr. Food Chem.* **4**, 418-425 (1956).
2. Private communication from Dr. WILLIS A. GORTNER, Pineapple Research Institute of Hawaii, Honolulu, Hawaii.
3. RABINOWITZ, J. C. AND SNELL, E. E., *Anal. Chem.*, **19**, 277-280 (1947).
4. TEPLY, L. J., DERSE, P. H., KRIEGER, C. H. AND ELVEHJEM, C. A., *J. Agr. Food Chem.*, **1**, 1204-1207 (1953).

* The National Canners Association and Dr. Willis A. Gortner, Pineapple Research Institute of Hawaii assisted in carrying out this work. This publication is No. XLVI in a National Canners Association series on The Nutritive Values of Canned Foods.

INSTITUTE of FOOD TECHNOLOGISTS
SCIENTIA ET MACHINA